(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 June 2006 (29.06.2006)

PCT

(10) International Publication Number
WO 2006/066416 A1

(51) International Patent Classification:
C12P 21/04 (2006.01) *C12Q 1/04* (2006.01)
C12Q 1/25 (2006.01)

(21) International Application Number:
PCT/CA2005/001972

(22) International Filing Date:
23 December 2005 (23.12.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/638,841 23 December 2004 (23.12.2004) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

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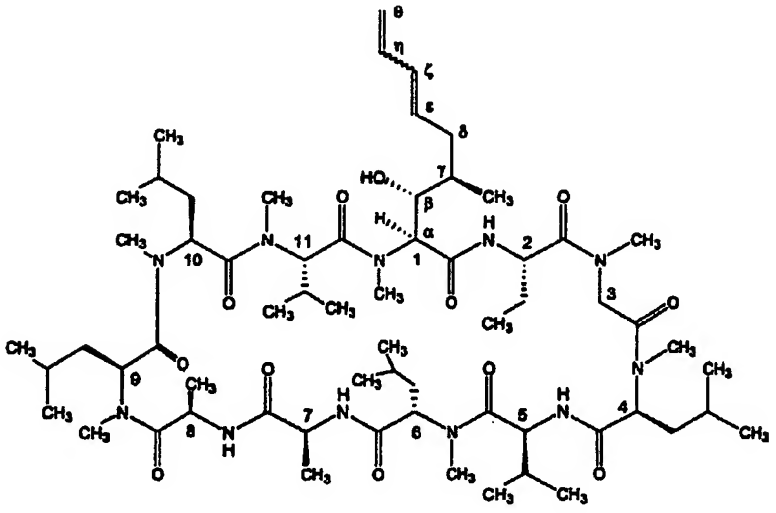
(54) Title: METHOD FOR BIOTRANSFORMATION OF THE CLYCLOSPORIN COMPOUND ISA247



(57) Abstract: A method of producing metabolites of a xenobiotic compound by biotransformation using a microorganism, wherein the xenobiotic compound is cyclosprorm ISA247, which is delivered to the microorganism in a mixture with a surfactant The method can be scaled up to produce large quantities of metabolites by, for example, biotransformation in a reactor The metabolites produced by the present method can be used for antibody production, as standards in therapeutic dose monitoring, or in pharmaceutical applications

WO 2006/066416 A1

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METHODS FOR THE BIOTRANSFORMATION OF THE CLYCLOSPORIN COMPOUND ISA247.

TECHNICAL FIELD

[0001] This invention relates to methods of preparing metabolites of compounds, and more particularly to the preparation of such metabolites by biotransformation.

BACKGROUND

[0002] When dosing a patient with a pharmaceutical compound, it is often necessary to monitor the serum levels of the drug to ensure that the patient is receiving a therapeutic dose of the drug. This is called therapeutic dose monitoring (TDM). TDM may measure the parent compound and/or one or more metabolites of the parent compound. Metabolites are formed when enzymes, commonly liver enzymes, work to break down or modify a drug so that the drug can be more easily eliminated from the body. When the parent compound is rapidly metabolized, it may be most convenient to measure the levels of a metabolite for the purposes of TDM. Frequently, immunoassays are used for such measurements.

[0003] When a TDM assay for measuring a metabolite is an immunoassay, the drug, or an isolated, purified metabolite of the drug, may be used for generating and/or selecting for an antibody having the desired specificity for use in that assay. Alternatively, the purified metabolite may be used to define an antibody specific for the parent compound, *i.e.*, an antibody that exhibits minimal cross-reactivity between the parent compound and metabolites of the parent compound. Therefore, efficient methods for producing isolated metabolites are needed in order to obtain a quantity of metabolite suitable for use in producing antibodies for TDM.

[0004] Metabolites may also have uses that are independent of TDM. Metabolites may have pharmaceutically important activities. For example, metabolites may exhibit beneficial characteristics such as improved pharmacokinetics, increased pharmacological activity or improved bioavailability. A metabolite of a parent drug compound may itself be a useful therapeutic. For example, A77 1726 is the active metabolite of leflunomide; hydroxy-tert-butylamide is the active metabolite of the HIV drug, nelfinavir; and 4-OH-tamoxifen is the active metabolite of tamoxifen. When the metabolite exhibits activity, efficient methods of producing

large quantities of the metabolite may be desired. Alternatively, one or more of the metabolites may be toxic. Thus, knowledge about how a drug is metabolized, its resulting metabolites, and the activity of these metabolites is important for understanding the activity of a drug. This information may also be required prior to drug approval. In order to identify the metabolites and their properties, a sufficient quantity of the metabolites must be produced and isolated.

[0005] One method for producing metabolites is to administer the drug to a mammal, such as a human, then collect blood, urine, bile or other body fluids, and extract, purify, and isolate metabolites from these fluids. Commonly, biotransformation, the conversion of a drug to metabolites of the drug, is achieved in human patients in the liver by the liver cytochrome P450 enzymes (CYP450 or P450). The P450 enyzme family includes an estimated 70 or so enzymes which act to render a compound more soluble for excretion in the bile or urine. To monitor metabolite formation by biotransformation, a parent compound may be tagged so that the metabolites may be recognized. Alternatively, a drug having a similar structure may be analyzed in parallel when the results are monitored by high pressure liquid chromatography separation and mass spectral analysis. A second method for biotransformation of a parent compound is to use a whole organ, a tissue slice, or cultured cells such as hepatocytes as a biotransforming system. In a third method, microsomes prepared from mammalian cells may be used. These approaches use animal isolates, thus risking introduction of unwanted contaminants into the metabolites. These methods are difficult to scale up when larger quantities of one or more of the metabolites is desired. In addition, biotransformations using microorganisms to convert the parent compound into metabolites may also be used.

[0006] It may be particularly difficult to produce large quantities of metabolites when the xenobiotic agent is highly lipophilic or highly insoluble in the aqueous media used in large scale fermentation methods. Therefore, a method for efficiently producing large quantities of metabolites of insoluble xenobiotics is needed.

SUMMARY

[0007] The present invention provides methods of producing metabolites of xenobiotic compounds by biotransformation using a microorganism. The xenobiotic compound may be

delivered to the microorganism in a mixture with a surfactant. The method can be scaled up to produce large quantities of metabolites by, for example, biotransformation in a reactor. The metabolites produced by the present method can be used, for example, for antibody production, as standards in therapeutic dose monitoring, or in pharmaceutical applications.

[0008] Accordingly, one aspect of the present invention provides a method for producing at least one metabolite of a xenobiotic compound in a microorganism, comprising the steps of:

(a) providing a mixture of the xenobiotic compound and a surfactant;
(b) adding the mixture to a culture of the microorganism; and
(c) incubating the culture for a period of time sufficient to allow the metabolite to form.

[0009] The mixture may comprise the xenobiotic compound, a solvent, and the surfactant. Any suitable solvent for the xenobiotic compound can be used. For example, the solvent may an alcohol, such as ethanol. The solvents may comprise more than one substance. In some embodiments, the solvent comprises both an alcohol and dimetheyl sulfoxide (DMSO).

[0010] The microorganism may be any microorganism that is capable of metabolizing the xenobiotic compound, preferably one that possesses the same metabolizing pathway for the xenobiotic compound as the human does. In certain embodiments, the microorganism is selected from the group consisting of *Actinoplanes sp., Streptomyces griseus, Streptomyces setonii*, and *Saccharopolyspora erthyraea*. The microorganism may also be *Cunningham ellaechinulata, Nerospora crassa*, or *Actinoplanes sp.*

[0011] The surfactant may be any suitable surfactant, which can be identified by a skilled artisan based on teachings of the present disclosure. For example, the surfactant may be selected from the group consisting of polyethylene glycol (PEG) 400, castor oil, isopropyl myristate, glycerine, Cremophor® (polyoxyl castor oil), Labrasol® (caprylocaproyl macrogolglycerides), and TWEEN® 40.

[0012] The xenobiotic compound is preferably a compound with a low solubility in aqueous solutions. In some embodiments, the xenobiotic compound is selected from the group consisting of immunosuppressants and anti-bacterial compounds, preferably a cyclosporin compound, more preferably ISA247 or cyclosporin A. The metabolite is preferably selected from the group

consisting of IM1-d-1, IM1-d-2, IM1-d-3, IM1-d-4, IM1-c-1, IM1-c-2, IM1-e-1, IM1-e-2, IM1-e-3, IM9, IM4, IM4n, IM6, IM46, IM69, and IM49.

[0013] The method of the present invention may optionally further comprise the step of isolating the metabolite from the culture.

[0014] Another aspect of the present invention provides a method for identifying a microorganism suitable for use in a biotransformation system comprising: a) comparing the structure of a compound to be metabolized with a known enzyme activity; b) identifying an enzyme that expresses the known enzyme activity; c) identifying a microorganism that expresses the identified enzyme; and d) using the microorganism that expresses the identified enzyme in a biotransformation system to make metabolites of the compound. In certain embodiments, the microorganism may be identified by comparing the genomic sequence of various microorganisms to the sequence of the identified enzyme, thereby identifying at least one microorganism that expresses the enzyme.

[0015] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

[0016] Figure 1 illustrates the structure of ISA247. The amino acid residues in ISA247 are indicated by numbers. Greek letters indicate the carbon positions of amino acid 1.

[0017] Figures 2A and 2B provide the structures of the *trans* (E-) and *cis* (Z-) isomers of ISA247 molecule, respectively.

[0018] Figure 3 is an HPLC scan showing the profile of the ISA247 metabolites isolated from human whole blood of a subject who had received a 50:50 mixture of *cis*:*trans* ISA247.

[0019] Figure 4 is an HPLC scan illustrating the profile of the ISA247 metabolites isolated from the biotransformation method described in Example 4.

[0020] Figure 5 is a graph showing the effects on ISA247 metabolite production of different surfactants in a biotransformation system.

[0021] Figure 6 shows the LC-MS profile of the ISA247 metabolites isolated from human whole blood of a subject who had received an ISA247 formulation that contained predominantly the *trans*-isomer of ISA247.

[0022] Figures 7 and 8 compare the effects of Media 3 and Media 16 on ISA247 metabolite production by *Actinoplanes sp.* (ATCC 53771) and *Saccharopolyspora erythraea* (ATCC 11635), respectively.

[0023] Figure 9 demonstrates the effects of different solvents and surfactants on ISA247 metabolite production by *Beauvaria bassiana*.

DETAILED DESCRIPTION

[0024] The present invention provides methods of producing metabolites of xenobiotic compounds by biotransformation using a microorganism. Specifically, the xenobiotic compound is delivered to the microorganism in a mixture with a surfactant. The method can be scaled up to produce large quantities of metabolites by, for example, biotransformation in a reactor. The metabolites produced by the present method can be used, for example, for antibody production, as standards in therapeutic dose monitoring, or in pharmaceutical applications.

[0025] Many pharmaceutically active compounds are poorly soluble in aqueous solutions. For example, cyclosporins and certain other immunosuppressive agents (rapamycin, azathioprine, mizoribine, and FK506 (tacrolimus)) are known to exhibit poor solubility in an aqueous environment. Using a cyclosporin derivative, ISA247, as a test compound, we discovered that microbial fermentation can be successfully used to prepare metabolites of poorly soluble compounds. Prior to describing the invention in further detail, the terms used in this application are defined as follows unless otherwise indicated.

Definition

[0026] The term "biotransformation," as used herein, refers to the process of metabolizing a compound by a living cell, particularly a cell of a microorganism.

[0027] A "xenobiotic compound," or "xenobiotic," is a compound that is not native with respect to a microorganism. A xenobiotic compound may be pharmaceutically active. The xenobiotic compounds of this invention are preferably not readily soluble in water. For example, the compound may have a water solubility, at 25°C, of 1 mg/ml or less, 0.75 mg/ml or less, 0.5 mg/ml or less, 0.25 mg/ml or less, 0.1 mg/ml or less, 0.08 mg/ml or less, 0.06 mg/ml or less, 0.04 mg/ml or less, or 0.02 mg/ml or less.

[0028] A "cyclosporin compound" is a cyclosporin, or derivative thereof, that has immunosuppressive activities. The term encompasses the naturally occurring cyclosporins, cyclosporin A to Z, ISA247, synthetic and artificial dihydro- and iso-cyclosporins such as those disclosed in US Pat. Nos. 4,108,985; 4,210,581 and 4,220,641; derivatized cyclosporins such as shown in US Pat. Nos. 4,384,996; 4,703,033; 4,764,503; 4,771,122; 4,798,823; 4,885,276; 5,525,590; 5,643,870; 5,767,069; and cyclosporine derivative compounds as provided in WO02069902; WO03033010; WO03030834; and WO04050687.

ISA247 and its metabolites

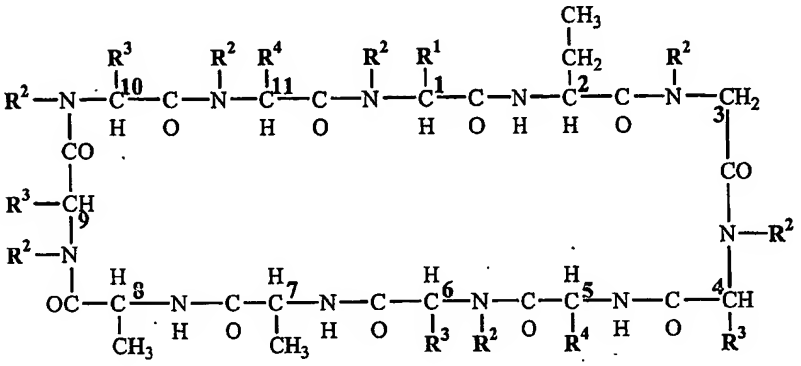
[0029] ISA247 (ISATX247 or ISA) and its ISA related family members are illustrated in US Pat. Nos. 6,613,739 and 6,605,593. Like cyclosporin A, ISA247 is a cyclic undecapeptide consisting almost entirely of hydrophobic amino acids. Many of these amino acids are not normally found in mammalian proteins. Figure 1 illustrates the structure of ISA247 and the 11 amino acid residues that comprise the cyclic peptide ring of this molecule. As shown, the amino acid residues are numbered in a clockwise direction. As shown in Figure 1, seven amino acids of the eleven amino acids of the 11-membered amino acid ring are N-methylated. The four remaining protonated nitrogen atoms can form intermolecular hydrogen bonds with carbonyl groups, which contribute substantially to the rigidity of the cyclosporin skeleton for both CsA and ISA247. CsA has a solubility of about 0.04 mg/ml at 25° C. Due to its low water-solubility,

the bioavailability of cyclosporin A is known to be 30% or less when orally administered to humans. ISA247 exhibits a similarly low water solubility.

[0030] ISA247 contains a sarcosine residue (whose three letter abbreviation is Sar; sarcosine is a methylated glycine residue and may also be abbreviated MeGly), one each of a D- and an L-alanine (Ala) residue, an α-amino butyric acid residue (Abu), a valine (Val) residue, an N-methyl valine (MeVal) residue, four N-methyl leucine (MeLeu) residues, and an alkene-containing 9-carbon, β-hydroxylated amino acid unique to the cyclosporins called (4R)-4-[(E)-2-butenyl]-4,N-dimethyl-L-threonine (MeBmt). The chemical name of ISA247 is cyclo{{(E)- and (Z)-(2S,3R,4R)-3-hydroxy-4-methyl-2-(methylamino)-6,8-nonodienoyl}-L-2-aminobutyryl-N-methyl-glycyl-N-methyl-L-leucyl-L-valyl-N-methyl-L-leucyl-L-alanyl-D-alanyl-N-methyl-L-leucyl-N-methyl-L-leucyl-N-methyl-L-valyl}. Its empirical formula is C63H111N11O12.I. It has a molecular weight of about 1214.85.

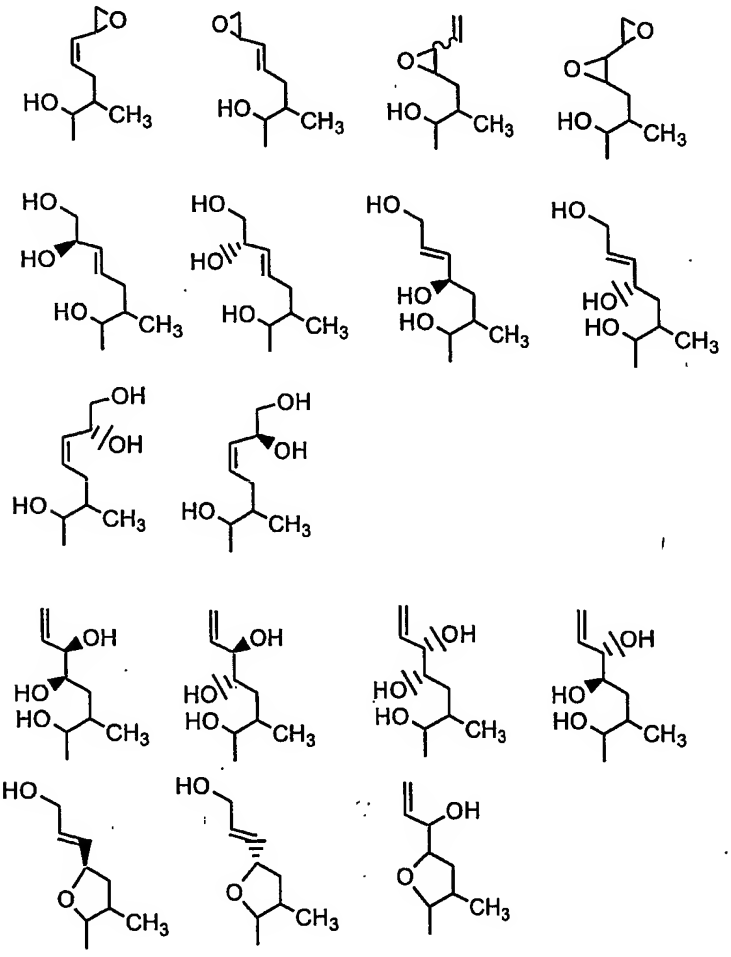
[0031] ISA247 is known to exist in two isomeric forms, *cis*-ISA247 (or Z-ISA247) and *trans*-ISA247 (or E-ISA247). Figures 2A and 2B illustrates the *trans* and *cis* forms of ISA247. A mixture of *cis* and *trans* forms of the ISA247 compound has been found to be less toxic and more potent than CsA (See U.S. Pat. Nos. 6,605,593 and 6,613,739). In addition, ISA247 has been found to be less toxic and more potent than CsA as a mixture of *cis* and *trans* forms when the mixture contains a predominant proportion of the *trans* isomer. When referring to ISA247, it will be understood by those of skill in the art that ISA247 is a mixture of the *cis* and *trans* isomers, and that the mixture may be enriched in the *trans* isomeric form of the compound. The isomeric compounds may be present in a mixture, ranging from 1:99 *cis*:*trans* to 99:1 *cis*:*trans*.

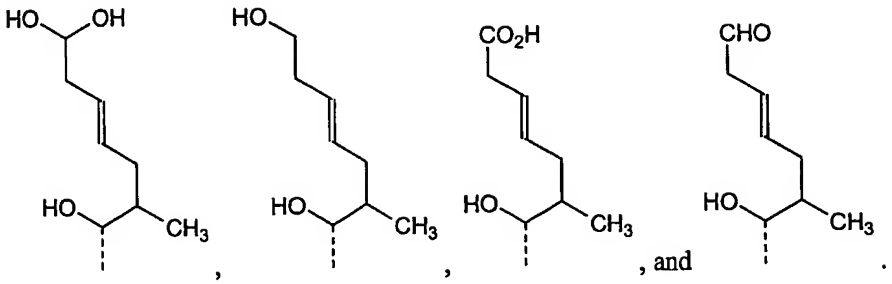
[0032] ISA247 metabolites can be described as follows: A compound of formula 1:



(I)

wherein R^1 is selected from the group consisting of:

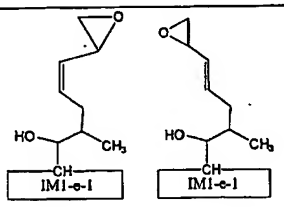
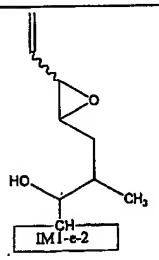


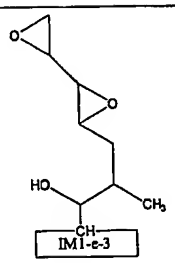
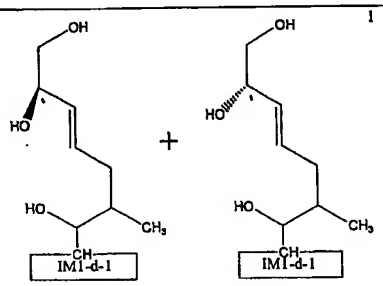
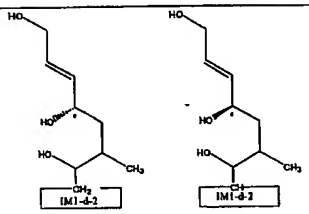
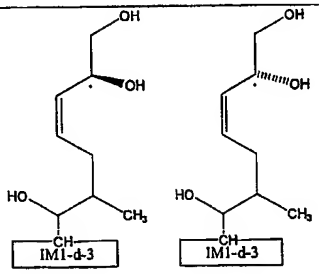
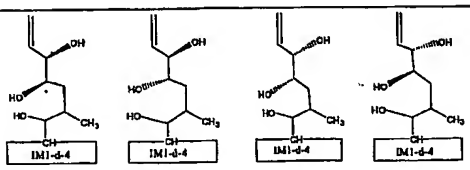
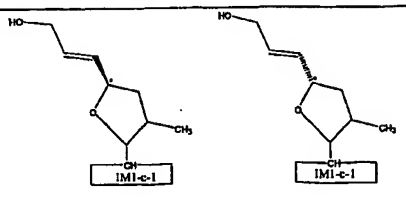


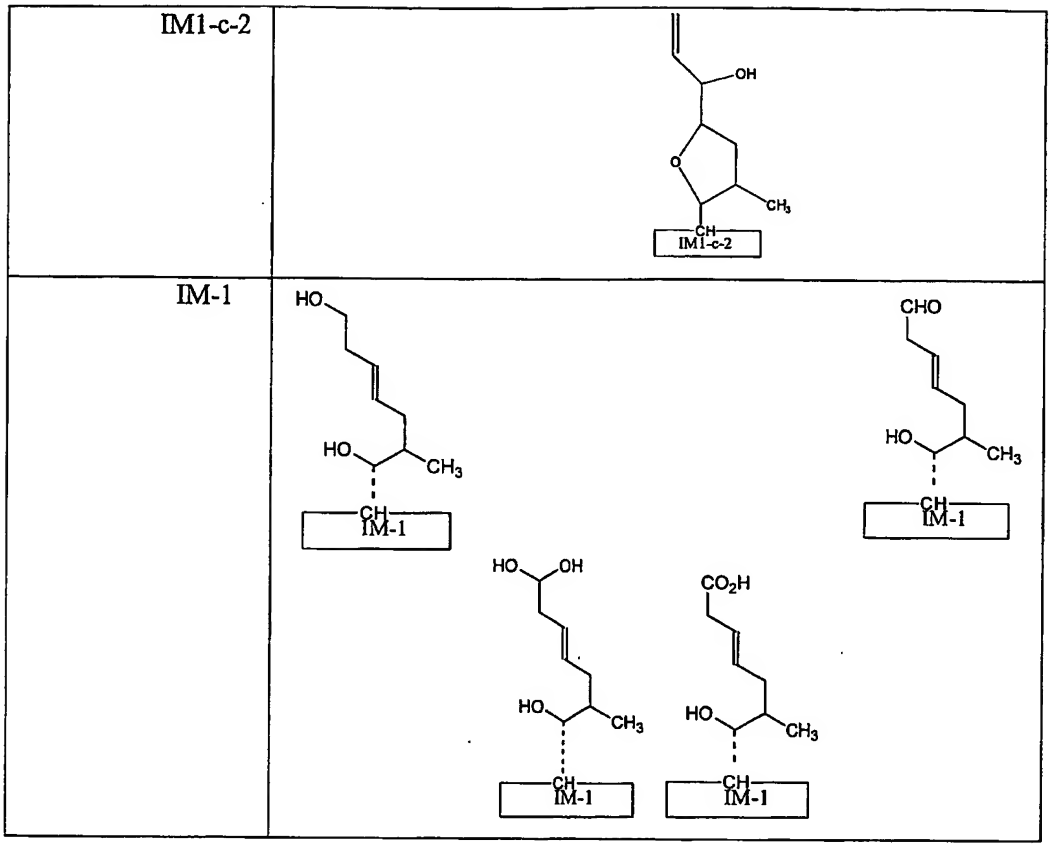
[0033] Where R^2 is selected from the group consisting of CH_3 and H; where R^3 is selected from the group consisting of CH2CH(CH3)2 and CH2C(CH3)2OH; and where R4 is selected from the group consisting of CH(CH3)2 and C(CH3)2OH.

[0034] Structures of ISA247 metabolites which are modifications at amino acid-1 of the ISA247 compound are illustrated in Table 1. The boxes represent amino acids 2-11 which form the ring portion of the cyclosporin structure with the modified amino acid-1, see Figure 1. Table 1 is not an exhaustive list of ISA247 metabolites that are modified at amino acid-1. For example, amino acid 1 metabolites may include 5, 6, 7 or 8 member rings.

Table 1: Amino Acid 1 Metabolites of ISA

IM1-e-1	
IM1-e-2	

IM1-e-3	
IM1-d-1	
IM1-d-2	
IM1-d-3	
IM1-d-4	
IM1-c-1	



[0035] ISA247 metabolites include N-demethylated metabolites where the N-demethylation occurs at at least one methylated nitrogen of the amide linkage of an amino acid, for example, IM4n, (or ISA247 Metabolite, N-demethylation at amino acid-4). N-demethylation can occur at amino acid-3 (IM3n), amino acid-4 (IM4n), amino acid-6 (IM6n), amino acid-9 (IM9n), amino acid-10 (IM10n) or amino acid-11 (IM11n). ISA247 metabolites also include hydroxylated metabolites where the hydroxylation occurs at at least one methyl leucine amino acid, for example amino acids 4, 6, 9 or 10 (IM4, IM6, IM9 or IM10), or at valine residue 5 (IM5) or at methyl valine residue 11 (IM11). IM46 are hydroxylated at both amino acids 4 and 6, IM49 are hydroxylated at both amino acids 4 and 9, and so on. Combinations of N-demethylated and hydroxylated metabolites can occur, as well as combinations of the metabolites which are alterations at amino acid-1, as shown in Table 1, with N-demethylations or hydroxylations. ISA247 metabolites also include metabolites which are glucuronide, sulfonide, glycosylated and

phosphorylated derivatives of hydroxylated metabolites of ISA247. U.S. Pat. Application No. ___ (Attorney Docket Number 16593-009001, filed December 19, 2005) co-pending and commonly assigned to the assignee provides ISA247 metabolites and uses thereof.

Methods of preparing ISA247 metabolites

[0036] Metabolites of ISA247 were first analyzed using human whole blood of a subject who had received a 50:50 mixture of *cis*:*trans* ISA247 (Example 1). Figure 3 is an HPLC scan illustrating the metabolite profile of metabolites isolated from the whole blood of this subject. Using organic extractions on human whole blood, metabolites were extracted, dried, reconstituted in methanol and identified using chromatographic techniques coupled with mass spectrometry. As shown in Figure 3, at least three diol , two hydroxylated and three N-demethylated metabolites were detectable in human whole blood.

[0037] A dog liver microsome preparation was also used to produce ISA247 metabolites (Example 2). While ISA247 metabolites can be produced in this manner, the yield was low and the cost was high. Therefore, it is not practical to obtain meaningful quantities of ISA247 metabolites using this approach.

[0038] To our knowledge, prior to the present invention, conventional biotransformation methods had not been reported as producing quantities of metabolites of CsA and ISA247, perhaps because of the lipophilic nature of these compounds. Without wishing to be bound by any theory, we believe the problem is that a hydrophobic compound, such as ISA247, has a tendency to adhere to the surfaces of filters, columns, and other hardware used to carry out the culture and process the product metabolites. Also, these highly lipophilic compounds do not go into solution in the aqueous environment of microorganisms in culture. Cultured microorganisms may not be able to access these lipophilic compounds to metabolize them. In some aspects, providing drug to a microbial growth preparation is not unlike providing drug to a mammal. A formulation that increases the bioavailability of the drug may be necessary.

[0039] In humans, cytochrome P450 enzymes are known to form metabolites from CsA. It has been found that cytochrome P450 enzymes also act to form ISA247 metabolites. Specifically, the cytochrome P450 enzyme CYP3A4 has been identified as the enzyme responsible for

cyclosporin and ISA247 metabolism. In order to produce a metabolite profile that is similar to that obtained from humans, the biotransformation system must utilize a microorganism which has the microbial equivalent of the human cytochrome P450 enzyme, grown in a medium and under culture conditions suitable for active growth and metabolism of the microorganism.

[0040] Biotransformation methods are exemplified in Smith *et al.* (Arch. Biochem. Biophys. (1974) 161: 551-558). Urlacker and Schmid (Curr Opin Biotechnol. (2002) 13(6):557-64) suggest that biotransformations could be performed using prokaryotic P450 monooxygenase enzymes. Venisetty andCiddi (Current Pharmaceutical Biotechnology (2003) 4(3):153-167) have proposed application of microorganisms to natural drugs to find novel drugs.

[0041] Biotransformation of cyclosporin A solubilized in methanol by *Sebekia benihana* to [γ-hydroxy-MeLeu]4 cyclosporin (AM4) and to [γ-hydroxy-MeLeu]^4 [γ-hydroxy-MeLeu]^6 cyclosporin (AM46) has been disclosed in US Pat. No. 6,255,100. However, it should be noted that when CsA is administered to a human, the predominate metabolites that are produced and monitored for TDM are AM1 (a metabolite of CsA that is hydroxylated at amino acid-1, MeBmt), AM9 (a metabolite that is hydroxylated at the MeLeu at amino acid position 9) and AM4n (a metabolite of CsA that is demethylated at the Meleu at amino acid position 4) (LeGatt *et al.*, Clin Biochem. (1994) 27(1):43-8). Thus, *Sebekia benihana* exemplifies a microorganism that does not provide a metabolic profile that mimics the mammalian metabolic profile of CsA.

[0042] We demonstrate herein that biotransformation can be used to produce metabolites of ISA247. As shown in Example 3, by delivering a mixture containing ISA247 and the surfactant TWEEN® 40 (polyoxyethylene sorbitan monopalmitate) to *Saccharopolyspora erytheraea*, all the main categories of ISA247 metabolites found in human blood were produced. Specifically, seven ISA247 metabolites were detected: IM4n (ISA247 Metabolite that is N-demethylated at amino acid-4), IM9 (ISA247 Metabolite that is hydroxylated at amino acid-9), IM4 (ISA247 Metabolite that is hydroxylated at amino acid-4), IM1-c-1(See table 1), IM1-d-1 (Table 1), IM1-d-2 (Table 1) and IM1-d-3 (Table 1). Different microorganisms produce different types, numbers and quantities of ISA247 metabolites, and the production can be optimized by changing the media (Example 5). Furthermore, for a given microorganism, the use of different surfactants or solvents may result in increased amount of metabolites or an improved production profile.

For example, PEG 400 and glycerol led to the production of greater amounts of metabolites when *Saccharopolyspora erytheraea* was used (Example 4), while TWEEN® 40 significantly increased the number of different metabolites produced by *Beauvaria bassiana* (Example 6).

[0043] Accordingly, one aspect of the present invention provides a method for producing at least one metabolite of a xenobiotic compound in a microorganism, comprising the steps of:

(a) providing a mixture of the xenobiotic compound and a surfactant;

(b) adding the mixture to a culture of the microorganism; and

(c) incubating the culture for a period of time sufficient to allow the metabolite to form. The method may optionally further comprise the step of isolating the metabolite from the culture.

[0044] Certain embodiments of the present invention provide an *in vitro* biotransformation system for producing significant quantities of metabolites of poorly soluble compounds such as those listed herein, especially immunosuppressive compounds such as cyclosporins (for example, ISA247 and CsA), macrolide lactones (for example, FK506), and triene macrolides (for example, rapamycin). Suitable xenobiotic compounds are discussed in greater detail below.

[0045] After addition of the parent compound-surfactant mixture to the bioreaction mixture that contains the microorganism in growth medium, the bioreaction is allowed to proceed for a time and under conditions which permit the parent compound to be metabolized. After the desired time, the metabolites are extracted from the bioreaction mixture, purified by separation, for example by chromatography such as high pressure liquid chromatography and mass spectral analysis (HPLC-MS). Nuclear magnetic resonance analysis may be used to verify that the individual metabolites have been isolated from one another and to verify the structure thereof. Individual metabolites that have been verified as separate chemical entities may be used as standards in subsequent assays.

[0046] A purified metabolite may be used in a TDM assay. For example, ISA247 may be administered to an organ transplant patient in a dose sufficient to achieve immunosuppression and prevent the rejection of a transplanted organ. In order to ensure that the patient is maintaining the proper drug level, and therefore maintaining the proper level of immunosuppression to prevent the rejection of a transplanted organ, a blood sample may be obtained from the patient at intervals. Blood levels of ISA247 may be measured. In addition,

blood levels of at least one metabolite may be monitored to ensure that the patient's body is metabolizing the drug in a predictable manner. If the patient's own metabolism is not working to eliminate the drug from the patient's system, blood levels of the unmetabolized, active drug may build up, requiring a change in the patient's dosing regimen. Quantification may be achieved by, for example, immunoassay or by HPLC-MS. Similarly, antibodies specific for ISA247 or one of its metabolites may be developed.

[0047] In some embodiments of the present invention, ISA247 in ethanol is mixed with glycerol and then added to a biotransformation system containing *Saccharopolyspora erytheraea* (*e.g.*, ATCC 11635). In other embodiments PEG 400 is mixed with ISA247 in ethanol prior to the addition of ISA247 to the biotransformation system. In other embodiments castor oil is mixed with ISA247 in ethanol prior to the addition of ISA247 to the biotransformation system. In other embodiments isopropyl myristate is mixed with ISA247 in ethanol prior to the addition of ISA247 to the biotransformation system. In other embodiments Cremophor® is mixed with ISA247 in ethanol prior to the addition of ISA247 to the biotransformation system. In other embodiments Labrasol® is mixed with ISA247 in ethanol prior to the addition of ISA247 to the biotransformation system. In other embodiments TWEEN® 40 is mixed with ISA247 in ethanol prior to the addition of ISA247 to the biotransformation system.

Additional exemplary xenobiotic compounds

[0048] Additional examples of drugs which are poorly soluble in aqueous solutions include: analgesics/antipyretics (*e.g.*, aspirin, acetaminophen, ibuprofen, naproxen sodium, buprenorphine, propoxyphene hydrochloride, propoxyphene napsylate, meperidine hydrochloride, hydromorphone hydrochloride, morphine, oxycodone, codeine, dihydrocodeine bitartrate, pentazocine, hydrocodone bitartrate, levorphanol, diflunisal, trolamine salicylate, nalbuphine hydrochloride, mefenamic acid, butorphanol, choline salicylate, butalbital, phenyltoloxamine citrate, diphenhydramine citrate, methotrimeprazine, cinnamedrine hydrochloride, and meprobamate); antiasthamatics (*e.g.*, ketotifen and traxanox); antibiotics (*e.g.*, neomycin, streptomycin, chloramphenicol, cephalosporin, ampicillin, penicillin, tetracycline, and ciprofloxacin); antidepressants (*e.g.*, nefopam, oxypertine, doxepin, amoxapine, trazodone, amitriptyline, maprotiline, phenelzine, desipramine, nortriptyline, tranylcypromine,

fluoxetine, doxepin, imipramine, imipramine pamoate, isocarboxazid, trimipramine, and protriptyline); antidiabetics (*e.g.*, biguanides and sulfonylurea derivatives); antifungal agents (*e.g.*, griseofulvin, ketoconazole, itraconizole, amphotericin B, nystatin, and candicidin); antihypertensive agents (*e.g.*, propanolol, propafenone, oxyprenolol, nifedipine, reserpine, trimethaphan, phenoxybenzamine, pargyline hydrochloride, deserpidine, diazoxide, guanethidine monosulfate, minoxidil, rescinnamine, sodium nitroprusside, rauwolfia serpentina, alseroxylon, and phentolamine); anti-inflammatories (*e.g.*, (non-steroidal) indomethacin, ketoprofen, flurbiprofen, naproxen, ibuprofen, ramifenazone, piroxicam, (steroidal) cortisone, dexamethasone, fluazacort, celecoxib, rofecoxib, hydrocortisone, prednisolone, and prednisone); antiteoplastics (*e.g.*, cyclophosphamide, actinomycin, bleomycin, daunorubicin, doxorubicin, epirubicin, mitomycin, methotrexate, fluorouracil, carboplatin, carmustine (BCNU), methyl-CCNU, cisplatin, etoposide, camptothecin and derivatives thereof, phenesterine, paclitaxel and derivatives thereof, docetaxel and derivatives thereof, vinblastine, vincristine, tamoxifen, and piposulfan); antianxiety agents (*e.g.*, lorazepam, prazepam, chlordiazepoxide, oxazepam, clorazepate dipotassium, diazepam, hydroxyzine pamoate, hydroxyzine hydrochloride, alprazolam, droperidol, halazepam, chlormezanone, and dantrolene); antimigraine agents (*e.g.*, ergotamine, propanolol, isometheptene mucate, and dichloralphenazone); sedatives/hypnotics (*e.g.*, barbiturates such as pentobarbital, pentobarbital, and secobarbital; and benzodiazapines such as flurazepam hydrochloride, triazolam, and midazolam); antianginal agents (*e.g.*, beta-adrenergic blockers; calcium channel blockers such as nifedipine, and diltiazem; and nitrates such as nitroglycerin, isosorbide dinitrate, pentaerythritol tetranitrate, and erythrityl tetranitrate); antipsychotic agents (*e.g.*, haloperidol, loxapine succinate, loxapine hydrochloride, thioridazine, thioridazine hydrochloride, thiothixene, fluphenazine, fluphenazine decanoate, fluphenazine enanthate, trifluoperazine, chlorpromazine, perphenazine, lithium citrate, and prochlorperazine); antiarrhythmics (*e.g.*, bretylium tosylate, esmolol, verapamil, amiodarone, encainide, digoxin, digitoxin, mexiletine, disopyramide phosphate, procainamide, quinidine sulfate, quinidine gluconate, quinidine polygalacturonate, flecainide acetate, tocainide, and lidocaine); antiarthritic agents (*e.g.*, phenylbutazone, sulindac, penicillamine, salsalate, piroxicam, azathioprine, indomethacin, meclofenamate, gold sodium thiomalate, ketoprofen, auranofin, aurothioglucose, and tolmetin sodium); antigout apents (*e.g.*, colchicine, and allopurinol); anticoagulants (*e.g.*, heparin, heparin sodium, and warfarin sodium); thrombolytic agents (*e.g.*, urokinase,

streptokinase, and alteplase); antifibriolytic agents (*e.g.*, aminocaproic acid); hemorheologic agents (*e.g.*, pentoxifylline); antiplatelet agents (*e.g.*, aspirin); anticonvulsants (*e.g.*, valproic acid, divalproex sodium, phenytoin, phenytoin sodium, clonazepam, primidone, phenobarbitol, carbamazepine, amobarbital sodium, methsuximide, metharbital, mephobarbital, mephenytoin, phensuximide, paramethadione, ethotoin, phenacemide, secobarbitol sodium, clorazepate dipotassium, and trimethadione); antiparkinson agents (*e.g.*, ethosuximide); antihistamines/antipruritics (*e.g.*, hydroxyzine, diphenhydramine, chlorpheniramine, brompheniramine maleate, cyproheptadine hydrochloride, terfenadine, clemastine fumarate, triprolidine, carbinoxamine, diphenylpyraline, phenindamine, azatadine, tripelennamine, dexchlorpheniramine maleate, methdilazine, and); antibacterial agents (*e.g.*, amikacin sulfate, aztreonam, chloramphenicol, chloramphenicol palmitate, ciprofloxacin, clindamycin, clindamycin palmitate, clindamycin phosphate, metronidazole, metronidazole hydrochloride, gentamicin sulfate, lincomycin hydrochloride, tobramycin sulfate, vancomycin hydrochloride, polymyxin B sulfate, colistimethate sodium, and colistin sulfate); antiviral agents (*e.g.*, interferon alpha, beta or gamma, zidovudine, amantadine hydrochloride, ribavirin, and acyclovir); antimicrobials (*e.g.*, cephalosporins such as cefazolin sodium, cephradine, cefaclor, cephapirin sodium, ceftizoxime sodium, cefoperazone sodium, cefotetan disodium, cefuroxime e azotil, cefotaxime sodium, cefadroxil monohydrate, cephalexin, cephalothin sodium, cephalexin hydrochloride monohydrate, cefamandole nafate, cefoxitin sodium, cefonicid sodium, ceforanide, cefadroxil, cephradine, and cefuroxime sodium; penicillins such as ampicillin, amoxicillin, penicillin G benzathine, cyclacillin, ampicillin sodium, penicillin G potassium, penicillin V potassium, piperacillin sodium, oxacillin sodium, bacampicillin hydrochloride, cloxacillin sodium, ticarcillin disodium, azlocillin sodium, carbenicillin indanyl sodium, penicillin G procaine, methicillin sodium, and nafcillin sodium; erythromycins such as erythromycin ethylsuccinate, erythromycin, erythromycin estolate, erythromycin lactobionate, erythromycin stearate, and erythromycin ethylsuccinate; and tetracyclines such as tetracycline hydrochloride, doxycycline hyclate, and minocycline hydrochloride, azithromycin, clarithromycin); anti-infectives (*e.g.*, GM-CSF); bronchodilators (*e.g.*, sympathomimetics such as epinephrine hydrochloride, metaproterenol sulfate, terbutaline sulfate, isoetharine, isoetharine mesylate, isoetharine hydrochloride, bitolterolmesylate, isoproterenol hydrochloride, terbutaline sulfate, epinephrine bitartrate, metaproterenol sulfate, epinephrine, and epinephrine bitartrate;

anticholinergic agents such as ipratropium bromide; xanthines such as aminophylline, dyphylline, metaproterenol sulfate, and aminophylline; mast cell stabilizers such as cromolyn sodium; steroidal compounds and hormones (*e.g.*, androgens such as danazol, testosterone cypionate, fluoxymesterone, ethyltestosterone, testosterone enathate, methyltestosterone, fluoxymesterone, and testosterone cypionate; estrogens such as estradiol, estropipate, and conjugated estrogens; progestins such as methoxyprogesterone acetate, and norethindrone acetate; corticosteroids such as triamcinolone, betamethasone, betamethasone sodium phosphate, dexamethasone, dexamethasone sodium phosphate, dexamethasone acetate prednisone, methylprednisolone acetate suspension, triamcinolone acetonide, methylprednisolone, prednisolone sodium phosphate, methylprednisolone sodium succinate, hydrocortisone sodium succinate, triamcinolone hexacetonide, hydrocortisone, hydrocortisone cypionate, prednisolone, fludrocortisone acetate, paramethasone acetate, prednisolone tebutate, prednisolone acetate, prednisolone sodium phosphate, and hydrocortisone sodium succinate; and thyroid hormones such as levothyroxine sodium); hypoglycemic agents (*e.g.*, glyburide, chlorpropamide, tolbutamide, and tolazamide); hypolipidemic agents (*e.g.*, clofibrate, dextrothyroxine sodium, probucol, pravastitin, atorvastatin, lovastatin, and niacin); antiulcer/antireflux agents (*e.g.*, famotidine, cimetidine, and ranitidine hydrochloride); antinauseants/antiemetics (*e.g.*, meclizine hydrochloride, nabilone, prochlorperazine, dimenhydrinate, promethazine hydrochloride, thiethylperazine, and scopolamine); and oil-soluble vitamins. Metabolites of these poorly soluble compounds may be produced using the methods of the instant invention.

Microorganisms

[0049] Suitable microorganisms for a successful biotransformation may be chosen based on the presence of microbial enzymes, such as cytochrome P450 enzymes, having the capacity to metabolize the parent compound. Microorganisms that may be useful for biotransformation methods include bacteria, fungi and actinomycetes which possess cytochrome P450 activity. The organisms having these enzymes can be identified empirically by comparing the metabolites found in the blood or urine after ISA247 administration with those found using a biotransformation or microbial conversion preparation. For example, CYP3A4 is a human P450 enzyme that can be characterized by its ability to hydroxylate testosterone, thereby producing 6β-hydroxytestosterone. The enzyme is inhibited by such compounds as clotrimazole, and

naringenin. It is induced by carbamazipine, phenobarbital, and rifampin. An organism, growing in growth media, which expresses an enzyme which has cytochrome P450 activity, should produce 6β-hydroxytestosterone when testosterone is introduced into the media, and this production should be affected by the known inhibitors and inducers. Other substrates metabolized by CYP3A4 include, for example, acetaminophen, diazepam, theophylline, warfarin, taxol, and nifedipine. Similarly, when these compounds are introduced into media containing a microorganism, if the microorganism expresses the enzyme, it should metabolize the substrate.

[0050] Known and characterized enzymes have known and characterized activity. By comparing the structure of the compound to be metabolized with the known activities of enzymes, enzymes can be identified that will be active in metabolizing the compound. Microorganisms can be screened for the presence of the identified enzyme. Thus, an aspect of the present invention provides a method of identifying a microorganism suitable for use in a biotransformation system where the method has the steps: a) comparing the structure of a compound to be metabolized with a known enzyme activities; b) identifying an enzyme that expresses the desired enzyme activity; c) identifying a microorganism that expresses the identified enzyme; and d) using the microorganism that expresses the identified enzyme in a biotransformation process to make metabolites of the compound. By using this method, microorganisms which may be useful to produce metabolites of a compound can be identified.

[0051] Alternatively, genetic sequence data may be used to identify potentially useful organisms by comparing the genomic sequence of an organism to the sequence of a known mammalian gene which encodes a cytochrome enzyme, for example CYP3A4. Microorganisms which have the appropriate genetic sequences, grown in the proper conditions, should express the target enzyme. In addition to reference compounds, compounds that inhibit or induce a particular human P450 enzyme can be tested in both systems.

[0052] Drugs which are known to be metabolized by specific cytochrome enzymes include: (1) acetaminophen, aromatic amines, caffeine, estradiol, imipramine, phenacetin, theophylline and warfarin, broken down by CYP1A2; (2) amitryptiline, bufurolol, captropril, clozepine, debrisopuine, flecainide, fluoxetine, haloperidol, metoprolol, mexiletine, sparteine, timolol,

tomoxetine, propranolol and codeine, broken down by CYP2D6; (3) acetaminophen, diazepam, amiodarone, benzphetamine, carbemazepine, cyclosporine, digitoxin, diltiazem, erythromycin, etopiside, flutamide, imipramine, lidocaine, loratidine, nifedipine, midazolam, retinoic acid, steroids, tamoxifen, taxol, terfenadine, THC, verapamil and warfarin, broken down by CYP3A4.

[0053] Microorganisms that express the CYP3A4 enzyme, and that may be useful for biotransformation methods include but are not limited to: *Actinoplanes sp.* (*e.g.*, ATCC No. 53771), *Streptomyces griseus* (*e.g.e.g.*, ATCC 13273), *Saccharopolyspora erythraea* (*e.g.*, ATCC No. 11635), and *Streptomyces setonii* (*e.g.*, ATCC No. 39116). Other useful microorganisms that may express cytochrome P450 enzymes include Amycolata *autotrophica* (*e.g.*, ATCC No. 35204), *Streptomyces californica* (*e.g.*, ATCC No. 15436), *Saccharopolysora hirsute* (*e.g.*, ATCC No. 20501), *Streptomyces* lavandulae (*e.g.*, ATCC No. 55209), *Stretomyces aureofaciens* (*e.g.*, ATCC No. 10762), *Streptomyces rimosus* (*e.g.*, ATCC No. 28893), *Bacillus subtillis* (*e.g.*, ATCC No. 55060), and *Nocardia asteroids* (*e.g.*, ATCC No. 3318), *Saccharomyces cerevisiae* (*e.g.*, ATCC No. 20137 or ATCC No. 64667) *Aspergillus nidulans* (*e.g.*, ATCC No. 32353) *Cunninghamella echinulata* var. *elegans* (*e.g.*, ATCC No.36112), Rhizopus stolonifer, (*e.g.*, ATCC No. 6227b), *Candida apicola* (*e.g.*, ATCC No. 96134), *Coprinus cireneus*, (*e.g.*, ATCC No. MYA-727, MYA-726, MYA-728, MYA-729, MYA-730, MYA-731).

[0054] Selection of appropriate culture time, culture conditions, extraction and purification methods is known to those of skill in the art. Growth of the chosen organism may be achieved by a skilled artisan, for example, by the use of an appropriate growth medium containing nutrients such as carbon and nitrogen, a buffering system, and trace elements and of conditions of pH, temperature, and aeration conducive to growth. Exemplary carbon sources include glucose, maltose, dextrin, starch, lactose, sucrose, molasses, soybean oil, and the like. Suitable nitrogen sources include soybean meal, cotton seed meal, fish meal, yeast, yeast extract, peptone, rice bran, meat extract, ammonium nitrate, ammonium sulfate and the like. Inorganic salts may be added such as phosphates, sodium chloride, calcium carbonate and the like. Different growth media may be used depending upon the stage of growth of the organism.

[0055] Exemplary conditions and media for growth of microorganisms suitable for use in the bioconversion of cyclosporins and cyclosporine derivatives thereof by *Saccharopolyspora erythraea* (for example, ATCC 11635), *Saccharopolysora hirsute* (for example, ATCC 20501), *Amycolata autotrophica* (for example, ATCC 35204), are provided in Corconan, Methods in Enzymology 43: 487-498 (1975), US Pat. Nos. 5,124,258; 6,043,064; and 6,331,622. Conditions for growth of Actinoplanes sp. (for example, ATCC No. 53771) are exemplified in US Pat. No. 5,270,187.

[0056] Exemplary growth conditions for microbial bioconversion of macrolides to their hydroxylated and/or demethylated metabolites include: 1) growth conditions as disclosed for the demethylation of L-679,934 (FK-506) to its metabolite, L-683,519, using *Actinomycete sp.* (Merck Culture Collection MA 6474; ATCC No. 53828) exemplified in US Pat. No. 5,268,370; and 2) demethylation of L-679,934 to L-682,993 or L-683,590 to L-683,742) by *Actinoplanes sp.* (ATCC No. 53771) using conditions for growth provided in US Pat. No. 5,202, 258. *Actinoplanes spp.* ATCC 53771, *Saccharopolyspora erthyreae* ATCC 11653, *Streptomyces lavandulae* ATCC 55209, *Stretomyces aureofaciens* ATCC 10762, *Streptomyces rimosus* ATCC 28893, *Bacillus subtillis* ATCC 55060 and *Nocardia asteroids* ATCC 3318 may be used to produce hydroxylated (for example, 24-OH rapamycin) and/or demethylated metabolites (for example, 39-O-demethylrapamycin) of rapamycin (Kuhnt, M., *et al.*, 1997, Enzyme and Microbial Technology 21: 405-412).

Surfactants

[0057] Suitable surfactants for use in an embodiment of the inventive method may be able to withstand autoclaving prior to being introduced into a microbial growth environment. Suitable surfactants are biocompatible surfactants and include but are not limited to nonionic surfactants such as polyethylene glycols for example PEG 300, PEG 400, PEG 600 (also known as Lutrol® E 300, Lutrol® E 400, Lutrol® E 600 Lutrol® F 127, and Lutrol® F 68 from BASF); caprylocaproyl macrogol-8 glycerides such as Labrasol® (Gatte Fosse, Cedex France); polyoxyethylene sorbitan fatty acid esters such as Tween® 20, Tween® 21, Tween® 40, Tween® 80, Tween® 80K, Tween® 81 and Tween® 85 (ICI Americas Inc., Bridgewater NJ, obtained from Aldrich Chemical Company Inc., Milwaukee Wis.); glycerine (BDH Fine

Chemicals, Toronto Ont.); castor oil (Wiler Fine Chemicals Ltd, London Ont.); Isopropyl myristate (Wiler Fine Chemicals Ltd, London Ont.); Cremophor® EL (Sigma Chemical, St Louis MO); and poloxamers such as Pluronics® F127 and Pluronics® L108 (BASF). Other surfactants that may be used include those that can act as lubricants or emulsifiers such as tyloxapol [4-(1,1,3,3-tetramethylbutyl)phenol polymer with formaldehyde and oxirane]; polyethoxylated castor oils such as Cremophor® A25, Cremophor® A6, Cremophor® EL, Cremophor® ELP, Cremophor® RH from BASF and Alkamuls EL620 from Rhone Poulenc Co; polyethoxylated hydrogenated castor oils, such as HCO-40; and polyethylene 9 castor oil.

[0058] Other surfactants that may be used include; polysorbate 20, polysorbate 60, and polysorbate 80; Cremophor® RH; poloxamers; Pluonics L10, L31, L35, L42, L43, L44, L61, L62, L63, L72, L81, L101, L121, L122; PEG 20 almond glyceride; PEG 20 corn glyceride; and the like. Suitable surfactants also include alkylglucosides; alkylmaltosides; alkylthioglucosides; lauryl macrogolglycerides; polyoxyethylene alkyl ethers; polyoxyethylene alkylphenols; polyethylene glycol fatty acids esters; polyethylene glycol glycerol fatty acid esters; polyoxyethylene-polyoxypropylene block copolymers; polyglycerol fatty acid esters; polyoxyethylene glycerides; polyoxyethylene sterols; polyoxyethylene vegetable oils; polyoxyethylene hydrogenated vegetable oils; polyoxyethylene alkylethers; polyethylene glycol fatty acids esters; polyethylene glycol glycerol fatty acid esters; polyoxyethylene sorbitan fatty acid esters; polyoxyethylene-polyoxypropylene block copolymers; polyglycerol fatty acid esters; polyoxyethylene glycerides; polyoxyethylene vegetable oils; polyoxyethylene hydrogenated vegetable oils; reaction mixtures of polyols such as PEG-10 laurate, PEG-12 laurate, PEG-20 laurate, PEG-32 laurate, PEG-32 dilaurate, PEG-12 oleate, PEG-15 oleate, PEG-20 oleate, PEG-20 dioleate, PEG-32 oleate, PEG-200 oleate, PEG-400 oleate, PEG-15 stearate, PEG-32 distearate, PEG-40 stearate, PEG-100 stearate, PEG-20 dilaurate, PEG-25 glyceryl trioleate, PEG-32 dioleate, PEG-20 glyceryl laurate, PEG-30 glyceryl laurate, PEG-20 glyceryl stearate, PEG-20 glyceryl oleate, PEG-30 glyceryl oleate, PEG-30 glyceryl laurate, PEG-40 glyceryl laurate, PEG-40 palm kernel oil, PEG-50 hydrogenated castor oil, PEG-40 castor oil, PEG-35 castor oil, PEG-60 castor oil, PEG-40 hydrogenated castor oil, PEG-60 hydrogenated castor oil, PEG-60 corn oil, PEG-6 caprate/caprylate glycerides, PEG-8 caprate/caprylate glycerides, polyglyceryl-10 laurate, PEG-30 cholesterol, PEG-25 phyto sterol, PEG-30 soya sterol, PEG-20

trioleate, PEG-40 sorbitan oleate, PEG-80 sorbitan laurate, polysorbate 20, polysorbate 80, POE-9 lauryl ether, POE-23 lauryl ether, POE-10 oleyl ether, POE-20 oleyl ether, POE-20 stearyl ether, tocopheryl PEG-100 succinate, PEG-24 cholesterol, polyglyceryl-10 oleate, sucrose monostearate, sucrose monolaurate, sucrose monopalmitate, PEG 10-100 nonyl phenol series, PEG 15-100 octyl phenol series, a poloxamer; PEG-35 castor oil, PEG-40 hydrogenated castor oil, PEG-60 corn oil, PEG-25 glyceryl trioleate, PEG-6 caprate/caprylate glycerides, PEG-8 caprate/caprylate glycerides, polysorbate 20, polysorbate 80, tocopheryl PEG-1000 succinate, and PEG-24 cholesterol, a poloxamer. In addition, oils such as almond oil; babassu oil; borage oil; blackcurrant seed oil; canola oil; coconut oil; corn oil; cottonseed oil; evening primrose oil; grapeseed oil; groundnut oil; mustard seed oil; olive oil; palm oil; palm kernel oil; peanut oil; rapeseed oil; safflower oil; sesame oil; shark liver oil; soybean oil; sunflower oil; hydrogenated castor oil; hydrogenated coconut oil; hydrogenated palm oil; hydrogenated soybean oil; hydrogenated vegetable oil; hydrogenated cottonseed and castor oil; partially hydrogenated soybean oil; soy oil; glyceryl tricaproate; glyceryl tricaprylate; glyceryl tricaprate; glyceryl triundecanoate; glyceryl trilaurate; glyceryl trioleate; glyceryl trilinoleate; glyceryl trilinolenate; glyceryl tricaprylate/caprate; glyceryl tricaprylate/caprate/laurate; glyceryl tricaprylate/caprate/linoleate; glyceryl tricaprylate/caprate/stearate; saturated polyglycolized glycerides; linoleic glycerides; caprylic/capric glycerides may be used. In addition, a mixture of surfactants and/or oils and/or alcohols may be used.

[0059] In some embodiments of the present invention, the selected lipophilic xenobiotic is mixed with an alkanol and a suitable nonionic surfactant before addition to an actively growing microbial culture. If the parent compound is mixed with an alcohol, the alcohol may be ethanol. Additional suitable alcohols include: methanol, isopropanol, 1-propanol, and other suitable alcohols well known in the art.

Solvents

[0060] In some embodiments of the present invention, the xenobiotic compound is mixed with a solvent before being added to a microorganism culture. The solvents may be sterilized prior to mixing with the xenobiotic compound. Optionally, a surfactant is also added to the xenobiotic compound – solvent mixture. Suitable solvents of the present invention may be any solvent that

does not inhibit the growth and metabolism of the microorganism. The solvent may be non-polar, polar aprotic, or polar protic. For example, the solvents include the hydrocarbon series solvents, such as benzene, toluene, n-hexane, cyclohexane, etc.; ether series solvents such as diethyl ether, tetrahydrofuran, 1,4-dioxane, methyl t-butyl ether, dimethoxyethane, ethylene glycol dimethyl ether, etc.; alcohols such as methanol, ethanol, 1-propanol, isopropanol, etc.; halogen-containing solvents such as methylene chloride, chloroform, 1,1,1-trichloroethane, etc.; and other solvents such as dimethylformamide, N-methylpyrrolidone, hexamethylphosphorotriamide, etc. The solvent is preferably not DMSO.

[0061] The following examples are offered to illustrate this invention and are not to be construed in any way as limiting the scope of the present invention. While this invention is particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

EXAMPLES

[0062] In the examples below, the following abbreviations have the following meanings. Abbreviations not defined have their generally accepted meanings.

°C	=	degree Celsius
hr	=	hour
min	=	minute
sec or s	=	second
μM	=	micromolar
mM	=	millimolar
M	=	molar
ml	=	milliliter
μl	=	microliter
mg	=	milligram
μg	=	microgram
mol	=	mole
pmol	=	picomole
ATCC	=	American Type Culture Collection
PBS	=	phosphate buffered saline
CSA	=	cyclosporin A
TDM	=	therapeutic dose monitoring
LC	=	liquid chromatography

MS = mass spectrometry
PEG = polyethylene glycol

General Material and Methods

Liquid Chromatographic (LC) Conditions

[0063] For Liquid Chromatography (LC or HPLC) a column having a stationary phase formed by chemically bonding a long-chain hydrocarbon group to a porous silica matrix, a Waters Symmetry C8, 2.1X50mm, 3.5µm analytical column (Waters cat# WAT 200624) with a guard column 2 x 20mm (Upchurch Scientific cat# C-130B) packed with Perisorb RP-8 (Upchurch Scientific cat# C-601) was used. The solvent percentages and flow rates utilized in the LC program are given in Table 2:

Time (min)	0.2% GAA + 10^{-5}M Na Acetate (%)	MeOH:MtBE (9:1) (%)	Flow rate (mL/min)
0.00	55	45	0.5
5.00	45	55	0.5
10.00	5	95	0.5
12.00	5	95	0.5
12.01	55	45	0.5
15.00	55	45	0.5

Mass Spectral (MS) Conditions

[0064] For Mass Spectroscopy, an Applied Biosystems / MDS Sciex API3000 (Analyst software v 1.2) machine was used. Run time was 15 minutes, injection volume was 5µL, Guard Column Temperature and Analytical Column Temperatures were 60°C. Manual settings were as follows: Turbo Ion Spray was 8000, Turbo Ion Spray horizontal setting was positive 4, Turbo Ion Spray lateral setting was 10. The Sciex machine was set with the parameters shown in Table 3.

Table 3 MS Settings

MS Settings:	
Scan type:	MRM (Multiple Reaction Monitoring)
Polarity:	Positive
Period Duration	15.00 min

Period Cycle:	1.32 sec
# of Cycles:	692
Advanced MS Settings:	
Resolution Q1:	Low
Q3:	Low
Intensity threshold:	0
Settling time:	50 msec
Pause time:	30 msec
Parameter Settings:	
Ion Source:	Turbo ion spray
Nebulizer Gas:	12
Curtain Gas:	8
Collision Gas:	12
Ion Spray voltage:	5000 V
Temperature:	550°C
Compound Settings:	
Declustering Potential:	60 V
Focusing Potential:	400 V
Collision Energy:	90 V

Table 4 shows ions and ion-specific instrument settings.

Q1 Mass (amu)	Q3 Mass (amu)	Time (msec)
1222.8	1098.7	100
1236.8	1112.7	100
1252.8	1128.7	100
1252.8	1224.7	100
1270.8	1112.7	100
1254..8	1130.7	100
1268.8	1128.7	100
1268.8	1144.7	100
1238.8	1114.7	100
1268.8	1240.8	100

Example 1

Preparation of ISA247 metabolites from whole blood

[0065] Whole blood was taken from humans after administration of ISA247 (a 50:50 mixture of *cis:trans* ISA247). ISA247 and its metabolites were extracted from whole blood using tertbutyl-methyl-ether (or methyl tertbutyl ether, MTBE), dried and reconstituted into methanol. 2mL of MTBE (cat. No. 7001-2; Caledon) were added to 200 uL of blood, shaken for 10 minutes, and spun down in a table top centrifuge for 2 minutes. The top MTBE layer was removed and concentrated under vacuum. That residue was reconstituted in 200 uL of methanol. Bile and urine extractions can be performed similarly, as can extractions from microsome preparations and biotransformation preparations. Once extracted, metabolites can be characterized using HPLC-MS, NMR, or other techniques known in the art. Figure 3 shows that results of LC-MS performed as described in General Materials and Methods. The ISA247 metabolites from whole blood include three main groups, the diols, hydroxylated and demethylated metabolites.

Example 2

ISA247 Metabolite Production by a Dog Liver Microsome Preparation

Preparation of Dog Microsomes

[0066] Dog liver microsomes were prepared in the following manner: after removing the liver, it was flushed with 1.15% potassium chloride (KCl); diced into small pieces (approximately 25 g) and ground until major chunks were disintegrated in chilled grinding buffer (0.1 M phosphate buffer pH 7.4; 4° C; 1:1 ratio of buffer to liver) utilizing a Polytron Homogenizer at 15,000 rpm for 3 to 5 minutes, thus forming a homogenate, which contained membrane-bounded organelles, including liver microsomes. After decantation of supernatant from the particulate matter, the supernatant was centrifuged for 90 min. at 100,000 x g to yield a pellet and a supernatant. Protein content was determined using the Lowry protein assay. The protein concentration of this microsomal preparation was approximately 23.2 mg/mL. To avoid enzyme activity loss, microsomes were stored in 4.0 or 6.0 mL aliquots at -80°C to avoid freeze thaw cycling.

[0067] The following ingredients were added stepwise into a 257 mL Erlenmeyer flask: 57.3 mg of NADP, 254 mg of Glucose-6-Phosphate, and 23.0 mg NADPH were added to 6.0 mL of Phosphate Buffer (adjusted to pH 7.4). Then, 2.0 mL of 5.0 mM MgCl, and 6.0 mL Glucose-6-Phosphate Dehydrogenase (10 units/mL, available from CALBIOCHEM, San Diege, CA, Cat. No. 346774) were added to the solution. Finally, 10 mL of Phosphate Buffer (pH 7.4) was added. A 6mL volume of dog liver microsome, prepared as above, was added into the flask, followed by ISA247, and incubated at 37°C for 2 hours at 250 rpm in an environmentally controlled incubator/shaker. At 2 hours, the reaction was stopped by adding 500 µL of 2M HCl.

[0068] Metabolites produced by this method were then extracted with an organic solvent, and further separated using high-pressure liquid chromatography (HPLC). The metabolites were further characterized by electrospray mass spectrometry (MS) and NMR. The resulting metabolite profile (data not shown) was similar to that from human whole blood. However, the diols from the dog microsome were much less abundant.

Example 3

ISA247 Metabolite Production by Biotransformation using *Saccharopolyspora erythraea*

[0069] This example illustrates a biotransformation system utilized microorganisms containing the microbial equivalent of human cytochrome P450 microsomal enzyme and a medium suitable for active growth of the microorganism. The parent compound, which is poorly soluble in water, was mixed with ethanol and a surfactant prior to addition to the biotransformation system. In this example, ISA247 in ethanol was mixed with TWEEN® 40 and then added to a biotransformation system containing *Saccharopolyspora erytheraea* (ATCC 11635).

[0070] Starting cultures were prepared as follows. Fifteen tubes (16 x 26 mm; 6 ml each) of the ISP2 medium slants were prepared containing 4 g/L yeast extract, 10g/L malt extract, 4g/L dextrose and 2g/L agar. These ingredients were mixed in demineralized water up to liter, pH neutralized as needed to 7 with NaOH. The medium was sterilized for 30 min. at 100° C. The tubes were stored at 4° C until use. Each slant was inoculated with *Saccharopolyspora erythraea*

(ATCC number 11635). Innoculated slants were grown for three weeks under sterile conditions at room temperature.

[0071] Precultures were transferred to Phase I Media. Phase I Media were prepared with 10 g/L dextrin, 1g/L glucose, 3 g/L beef extract, 10 g/L yeast extract, 5 g/L magnesium sulfate and 400 mg/L potassium phosphate. These ingredients were mixed in deionized water up to 1 liter, pH neutralized as needed to 7 with NaOH and 50 mL was aliquoted into each of two baffled 250 mL culture flasks. The medium was sterilized for 30 min. at 100° C. 5 mL of the media was aliquoted into a slant tube containing *Saccharopolyspora erythraea*. The cells were scraped off the surface of the slant and 2.5 mL of the suspension was placed in each flask. The flasks were placed on a Labline Incubator at 27° C and shaken at 250 rpm for 3 days (72 hrs).

[0072] *Saccharopolyspora erythraea* was transferred to Phase II media from Phase I media by centrifuging the contents of a Phase I flask at 3300 rpm for 5 min. and decanting off the supernatant to obtain a pellet. 5 mL of Phase II media was added to the pellet and the tube was vortexed, then centrifuged at 3300 rpm for 4 min. Again the supernatant was decanted. The pellet was resuspended in Phase II media. The subsequent suspension was added to 50 mL of Phase II medium in a baffled culture flask.

[0073] Phase II Media contained 10 g/L glucose, 1 g/L yeast extract, 1 g/L beef extract and 11.6 g/L of 3-N-morpholinopropanesulfonic acid (MOPS) buffer. These ingredients were mixed in deionized water to one liter; then 50 mL were dispensed into two baffled culture flasks (250 mL). After adjustment to pH to 7.0 with 5M NaOH, the medium was autoclaved for 30 min. at 100° C, then cooled. TWEEN® 40 was autoclaved before mixing with ISA247 and ethanol.

[0074] ISA247 (4mg of ~50/50 mixture of E and Z isomers) was dissolved in 95% ethanol (0.1 ml), then mixed with 0.4 ml TWEEN® 40 (polyoxyethylene sorbitan monopalmitate; Cat. No. P1504. Sigma-Aldrich, St. Louis, MO) The parent compound-surfactant mixture was then added to *Saccharopolyspora erythraea* in the Phase II culture medium. A zero time sample was obtained and frozen. Each flask was then capped and placed on an Innova Incubator at 27° C and incubated for 120 hrs with shaking at 170 rpm.

[0075] A second sample was obtained from the Phase II culture medium. The zero time sample and the second sample were extracted using tert-butyl-methyl ether (cat. No. 7001-2; Caledon). The extracted metabolites were reconstituted in methanol (HPLC grade) and analyzed by LC-MS as previously described. As shown in Figure 4, the metabolite profile obtained by this method is similar to that obtained from human whole blood (see Example 1). When the biotransformation mixture was analyzed, seven metabolite compounds were found to be present: IM4n (ISA247 Metabolite that is N-demethylated at amino acid-4), IM9 (ISA247 Metabolite that is hydroxylated at amino acid-9), IM4 (ISA247 Metabolite that is hydroxylated at amino acid-4), IM1-c-1(See table 1), IM1-d-1 (Table 1), IM1-d-2 (Table 1) and IM1-d-3 (Table 1). Therefore, seven out of eight ISA247 metabolites revealed in human blood were produced in this biotransformation system.

EXAMPLE 4

Effect of Varying the Surfactant in Biotransformation using *Saccharopolyspora erythraea*

[0076] To test the effects of different surfactants, actively growing Phase II cultures of *Saccharopolyspora erythraea* (ATCC number 11635) were prepared as above. Seven tubes each containing ISA247 (56 mg; 50/50 mixture of E- and Z-isomers) in ethanol (0.33 ml) were prepared. A surfactant (0.67 ml/tube) was added to each tube as follows:

Tube 1 – PEG 400 (polyethylene glycol 400; Carbowax – Fisher Scientific, FairLonn NJ);

Tube 2 – castor oil (Wiler Fine Chemicals Ltd, London Ont.);

Tube 3 – isopropyl myristate (Wiler Fine Chemicals Ltd, London Ont.);

Tube 4 – glycerine (BDH Fine Chemicals, Toronto Ont. Lot # 120343/73865);

Tube 5- Cremophor® EL (Sigma Chemical, St Louis MO);

Tube 6 – Labrasol® (Gatte Fosse, Cedex France); and,

Tube 7- TWEEN® 40 (Aldrich Chemical Company Inc., Milwaukee Wis.).

[0077] The parent compound-surfactant mixture was added to the actively growing culture of Saccharopolyspora and a zero time sample was taken. After incubation with shaking at 27°C for 5 days, samples were obtained, extracted, and the metabolites were quantified as described in Example 4. Area under the curve of HPLC peaks, similar to those shown in Figures 3 and 4, was measured as an indication of the quantity of metabolite present. The HPLC peaks corresponded to one N-demethylated metabolite, which was identified as IM4n; two hydroxylated metabolites which were identified as IM4 and IM9; one cyclic metabolite identified as IM1-c-1; and three diol metabolites, diols formed at the 1 amino acid of the ISA247 compound, identified as IM1-d-1, IM1-d-2 and IM1-d-3 (See Table 1). The seven surfactants were not all equivalent in their activity in increasing the production of metabolites in the biotransformation preparation. As shown in Figure 5, the addition of glycerine or PEG 400 to the biotransformation preparation resulted in significant increases in the quantity of metabolites produced. However, the addition of castor oil, isopropyl myristate, Cremophor®, Labrasol® and TWEEN® 40 all resulted in an increase in the production of metabolites in the biotransformation preparation, over the production of metabolites in the preparation without surfactant (not shown).

EXAMPLE 5

Biotransformation Using Various Microorganisms

[0078] A variety of microorganisms were evaluated for production of ISA247 metabolites from ISA247, including *Curvularia lunata* (University of Alberta Microfungal Collection and Herbarium (UAMH) 9191; ATCC 12017), *Cunninghamella echinulata* var. *elegans* (UAMH 7370; ATCC 36112), *Curvularia echinulata* var. *blakesleena* (UAMH 8718; ATCC 8688a), *Cunninghamella echinulata* var. *elegans* (UAMH 7369; ATCC 26269), *Beauvaria bassiana* (UAMH 8717; ATCC 7159), *Actinomycetes* (ATCC 53828), *Actinoplanes sp.* (ATCC 53771), *Cunninghamella echinulata* (UAMH 4144; ATCC 36190), *Cunninghamella echinulata* (UAMH 7368; ATCC 9246), *Cunninghamella bainiere* (*echinulata*) (UAMH 4145; ATCC 9244) and *Saccharopolyspora erythraea* (ATCC 11635).

[0079] These microorganisms were screened for metabolite conversion yield (amount of known ISA247 metabolites produced) as well as metabolic diversity (number of different

ISA247 metabolites produced). The microorganisms were grown in Phase I and incubated with ISA247 in Phase II. After the addition of ISA247 to the fermentation media, samples were taken from the media and analyzed with LC-MS against a human standard ISA247 metabolite profile to identify and quantify the metabolites collected. After primary testing of each strain via 96 hour biotransformation cycles, the two strains with the highest combination of metabolite conversion and metabolic diversity were tested again in Phase III with different media compositions, in order to select improved media compositions.

Phase I/Phase II methods

[0080] Each microorganism tested was maintained on culture-specific agar slants. All slants were prepared one month in advance to avoid contamination. Prepared agar media were autoclaved at 123°C and partial pressure of 360 mmHg for 58 minutes, cooled slightly, and 6 mL was pipetted into sterile 16x125 mm culture tubes. After placing the agar into the tube, the tube was rested on an incline to create a slant, cooled until the agar set, labeled, and incubated at 27°C for 1-2 weeks. ATCC 11635 and ATCC 53771 were sporulated using ISP agar (0.4% yeast extract, 1% malt extract, 0.4% dextrose and 2% granulated agar). ATCC 53828, UAMH 8717 and UAMH 8718 were sporulated using potato dextrose agar (PDA, 3.9% in distilled water). UAMH 4145, UAMH 7369, UAMH 7370 and UAMH 9191 were sporulated on cereal slants (10%% mixed cereal, dry, preferably pabulum for infants; 2% granulated agar; the cereal was mixed before and after sterilization to prevent clumping of the cereal and inadequate distribution of agar in the slants). Following incubation for two weeks, each microorganism was inoculated onto the microorganism-specific agar and returned to 27°C. Once a full lawn of colonies was seen on the slants, the slants were preferably used immediately in Phase I or, if necessary, stored at 4°C.

[0081] Subsequently, 2mL of sterile Phase I media Phase I media (containing ISP seed broth: 1% dextrin, 1% glucose, 0.27% beef extract, 1% yeast extract, 0.004% magnesium sulfate, and 0.036% potassium diphosphate, at pH 7.0) was added to a source slant containing the microorganism to be tested. Using a sterile inoculating loop, the colonies were removed, vortexed and then the resulting suspension was added to 50 mL of sterile Phase I media,

contained in a sterile, 250-mL baffled culture flask. Each flask was incubated for 96 hours in to increase biomass before the addition of ISA247 in Phase II.

[0082] To prepare biomass for transfer to Phase II, the cells were washed thoroughly to remove Phase I residue. Phase I contents were aseptically transferred into a 50-mL conical centrifuge tube, centrifuged at 3300 rpm for five minutes, and decanted to remove supernatant. The cells were washed with 5mL of excess Phase II media (3.65% MOPS, 0.31% yeast extract, 3.14% glucose and 0.31% beef extract, at pH 7.0) and centrifuged again for five minutes at 3300 rpm, after which the supernatant was decanted and 5mL of fresh Phase II media was added. The resulting mixture was vortexed thoroughly and quantitatively transferred under aseptic conditions to 50 mL of sterile Phase II media in a 250mL baffled culture flask. A portion of ISA247 (0.5 mL, 56 mg/mL of ISA247 that was predominantly in *trans* form in 33.75% ethanol (95%):66.25% glycerol) was added to the media and the mixture was shaken vigorously. Sample aliquots were taken (0.5mL, 12 hour increments over 96 hours of Phase II fermentation) and stored at -80°C until LC-MS analysis. Fermentation was concluded after 96 hours.

Phase III

[0083] In Phase III, ATCC 53771 and ATCC 11635 strains were incubated for 96 hours in Media C (2% glucose, 2% starch, 0.5% yeast extract, 2% soybean protein, 0.32% $CaCO_3$, 0.25% NaCl) as seed broth and then aseptically transferred in separate replicate trials to either Media 3 (2% glycerol, 0.5% peptone, 0.5% yeast extract, 0.2% beef extract, 0.1% $(NH_4)_2HPO_4$, 0.1% $CaCO_3$, 0.3% NaCl, 0.03% $MgSO_4$-$7H_2O$, at pH 7.0) or Media 16 (2% glucose, 1% glycerin, 1% peptone, 1% meat extract, 2% soybean protein, 0.5% $CaCO_3$, 0.5% NaCl, at pH 7.0). Care was exercised when taking samples for LC-MS due to the viscous consistency of the fermentation media.

Table 5: Summary of the media and agar used in Example 5

	ATCC 11635	ATCC 53771	ATCC 53828	UAMH 4145	UAMH 7369	UAMH 7370	UAMH 8717	UAMH 8718	UAMH 9191
Media	ISP 2	ISP 2	ISP 2	ISP 2	ISP 2	ISP 2	ISP 2	ISP 2	ISP2
	Media 3	Media 3							

	ATCC 11635	ATCC 53771	ATCC 53828	UAMH 4145	UAMH 7369	UAMH 7370	UAMH 8717	UAMH 8718	UAMH 9191
	Media 16	Media 16							
Agar	ISP	ISP	PDA	CER	CER	CER	PDA	PDA	CER

Metabolite analysis by LC-MS

[0084] Samples were thawed from storage at -80°C and 16x10 mm culture tubes were labeled to represent the samples to be analyzed. A 200 µL aliquot was removed from each 0.5 mL sample, and 25uL of a 1 mg/mL solution of CsA (Cyclosporine A) was added as an internal standard. 2mL of HPLC-grade methanol was added to each sample and the samples were capped and shaken for twenty minutes. The samples were centrifuged at 3300 rpm for 1minute and 45seconds. The supernatant was decanted into clean, labeled 16x10 mm culture tubes and vacuum concentrated to remove organic solvent. The dried layer, containing both the metabolites and parent drug was re-constituted in 200 µL of HPLC-grade methanol, and the samples were quantitatively transferred to auto sampler vials. Samples were run for 15 minutes in deionized water with 0.01% acetic acid/sodium acetate, starting with a 12 minute gradient of increasing m-TBE (methyl tert-butyl ether) and HPLC grade methanol.

[0085] Figure 6 is a graph of mass spec signal versus retention time for typical metabolites from a sample pooled from human participants. Table 6 summarizes the ion masses found, corresponding quantifiable ISA247 metabolites and approximate retention times. Ion masses quantified included 1223, 1237, 1239, 1253, 1255, 1267 and 1271. Note that two diols (IM1-d-1 and IM1-d-4) were detected here, whereas three diols (IM1-d-1, IM1-d-2, and IM1-d-3) were detected in Example.1. The reason for this discrepancy is that the *trans*-form of ISA247 is metabolized to IM1-d-1 and IM1-d-4, while the *cis*-form of ISA247 is metabolized to IM1-d-2 and IM1-d-3. Since the parent compound used in Example 1 was a 50:50 mixture of *cis*:*trans*, and the parent compound in this Example was predominantly *trans*-ISA247, the metabolite profiles were different with respect to the diols.

Table 6

Ion Mass	Metabolite from ISA247	Approximate Retention Time (min)
1223	IM4n	9.789
1237	ISA247	10.206
1239	1239	8.340
1253	IM1-c-1 ; IM9; IM4	8.575;8.939;9.440
1255	1255	8.899
1267	CSA(Internal Standard)	10.678
1271	IM1-d-1; IM1-d-4	7.535; 8.166

[0086] The relative % conversion was calculated from the ratio of the area under the curve (AUC) of the peak for each quantifiable metabolite detected and the area under the curve of the Internal CsA standard using the following equation:

% Conversion = (Metabolite AUC)/ (CsA Internal Standard AUC) x 100

Results

[0087] The metabolic diversity after 96 hours of biotransformation is summarized in Table 7. A check mark indicates a quantifiable amount of metabolite was produced. Table 8 lists the amount of each metabolite produced in each microorganism tested.

Table 7

Metabolite	ATCC 11635	UAMH 4145	ATCC 53771	ATCC 53828	UAMH 7369	UAMH 7370	UAMH 8717	UAMH 8718	UAMH 9191
IM1-d-1	√	√	√		√	√		√	√
IM1-d-4	√						√		√
1239	√		√	√		√	√	√	√
1255	√	√	√				√		
IM4n	√	√	√		√			√	√
IM1-c-1	√	√	√	√	√	√		√	√

Metabolite	ATCC 11635	UAMH 4145	ATCC 53771	ATCC 53828	UAMH 7369	UAMH 7370	UAMH 8717	UAMH 8718	UAMH 9191
IM9	√	√		√	√	√		√	√
IM4	√	√		√				√	
Rank	1st	4th	3rd	9th	8th	7th	6th	5th	2nd

Table 8 (All weights in nanograms)

Metabolite	ATCC 11635	UAMH 4145	ATCC 53771	ATCC 53828	UAMH 7369	UAMH 7370	UAMH 8717	UAMH 8718	UAMH 9191
IM1-d-1	1120.3	<1.00	<1.00	<1.00	<1.00	<1.00	0.00	<1.00	13.96
IM1-d-4	4.38	0.00	0.00	0.00	0.00	0.00	2.65	0.00	1.11
1239	6.38	<1.00	<1.00	<1.00	<1.00	<1.00	9.42	<1.00	2.44
1255	23.11	0.00	<1.00	0.00	0.00	0.00	<1.00	0.00	0.00
IM4n	22.02	<1.00	20.99	1.51	0.00	0.00	0.00	<1.00	7.01
IM1-c-1	71.80	<1.00	3.175	<1.00	<1.00	<1.00	0.00	<1.00	6.78
IM9	63.22	<1.00	0.00	<1.00	<1.00	<1.00	0.00	<1.00	11.69
IM4	35.40	<1.00	0.00	1.18	0.00	<1.00	0.00	<1.00	1.66

[0088] Thus, among the microorganisms examined in this experiment, ATCC 11635 displayed the greatest percent conversion and the greatest metabolic diversity. Eight known human ISA247 metabolites were detected in ATCC 11635 samples. UAMH 4145 produced six of the eight metabolites. ATCC 53771, often used in the lab because of its inherent ability to generate large amounts of IM4n (6.66%), produces five of the eight human metabolites. ATCC 53828 produced four of the eight metabolites; although each of these metabolites was produced in small quantities, the rare metabolite 1239 was produced. UAMH 7369 and UAMH 7370 each produced four of the metabolites. UAMH 9191 and UAMH 8718 each produced six metabolites. UAMH 8717 produced three metabolites.

[0089] Another factor considered in microorganism selection was the presence of "rare" metabolites of ISA247. "Rare" is defined as metabolites that are not produced in large amounts by ATCC 11635, *e.g.*, IM1-d-4, 1239 and 1255. IM1-d-4 was present in ATCC 11635, UAMH

8717 and UAMH 9191, but was produced in the greatest quantity by ATCC 11635. The microbial strains ATCC 11635, ATCC 53771, ATCC 53828, UAMH 7370, UAMH 8717, UAMH 8718, UAMH 9191 all produced 1239. The microorganism that produced the greatest quantity was UAMH 8717. The metabolite corresponding to ion 1255 was manufactured by ATCC 11635, UAMH 4145, ATCC 53771, UAMH 8717, with the greatest conversion by ATCC 11635.

[0090] In Phase III of this experiment, ATCC 11635 and ATCC 53771 were cultured in Media 3 and Media 16, and the effects of the media compared. Figure 7 shows the results for ATCC 11635. Media 3 and Media 16 produced similar amounts of each metabolite except for IM1-d-1, IM1-d-4 and IM1-c-1. IM1-d-1 production decreased with Media 3 and was not present in detectable levels with Media 16. IM1-d-4 production decreased with both Media 3 and Media 16. IM1-c-1 production increased 10% with Media 3. Figure 8 is a graph of the effect of media composition on the production of ISA247 metabolites in ATCC 53771. IM1-d-1 was detected only when using ISP2 media and IM9 and IM4 were only detected when using Media 3 and Media 16. Most of the metabolites, with the exception of IM1-d-4 were increased in quantity by using Media 3 and Media 16. Therefore, the growth media of the microorganisms can be altered to optimize the effect of biotransformation.

EXAMPLE 6

Effect of surfactants and solvents in biotransformation using *Beauvaria bassiana*

[0091] In Example 5, *Beauvaria bassiana* (UAMH 8717) only weakly produced ISA247 metabolites. To test if different surfactants or solvents can change the production profile, dimethyl sulfoxide (DMSO) and TWEEN® 40 were compared to glycerol (used in Example 5). 0.5 mL of a 56 mg/mL ISA247 solution (in 33.75% ethanol (95%):66.25% DMSO or TWEEN® 40 instead of glycerol) was added into 50 mL of media. The same Phase I and Phase II media and procedure of Example were followed.

[0092] As shown in Figure 9, the use of glycerol as a surfactant led to the formation of IM1-d-4, 1239, and 1255; DMSO led to the formation of IM1-d-1, IM4n and IM4; and TWEEN® 40 resulted in IM1-d-1, IM1-d-4, 1255, IM4n, IM9 and IM4. The amount of conversion was also dramatically changed. Consequently, biotransformation results can be optimized by changing

the solvents or surfactants used to deliver the parent compound, and a skilled artisan can choose a certain solvent, surfactant and media to increase the production of a given metabolite of combination of metabolites.

[0093] All of the publications, patents and patent applications cited in this application are herein incorporated by reference in their entirety to the same extent as if the disclosure of each individual publication, patent application or patent was specifically and individually indicated to be incorporated by reference in its entirety.

[0094] A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A method for producing at least one metabolite of a xenobiotic compound in a microorganism, comprising the steps of:

(a) providing a mixture of the xenobiotic compound and a surfactant;

(b) adding the mixture to a culture of the microorganism; and

(c) incubating the culture for a period of time sufficient to allow the metabolite to form.

2. The method of claim 1 wherein the mixture comprises the xenobiotic compound, a solvent, and the surfactant.

3. The method of claim 2 wherein the solvent is an alcohol.

4. The method of claim 3 wherein the alcohol is ethanol.

5. The method of any one of claims 1-4 wherein the solvent comprises both an alcohol and DMSO.

6. The method of any one of claims 1-5 wherein the microorganism is selected from the group consisting of *Actinoplanes sp., Streptomyces griseus, Streptomyces setonii*, and *Saccharopolyspora erthyraea.*

7. The method of any one of claims 1-5 wherein the microorganism is *Cunningham ellaechinulata.*

8. The method of any one of claims 1-5 wherein the microorganism is *Nerospora crassa.*

9. The method of any one of claims 1-5 wherein the microorganism is *Actinoplanes sp.*

10. The method of any one of claims 1-9 wherein the surfactant is selected from the group consisting of PEG 400, castor oil, isopropyl myristate, glycerine, cremophor®, Labrasol®, and TWEEN® 40.

11. The method of any one of claims 1-10 wherein said xenobiotic compound is selected from the group consisting of immunosuppressants and anti-bacterial compounds.

12. The method of any one of claims 1-11 wherein the xenobiotic compound is a cyclosporin compound.

13. The method of claim 12 wherein the cyclosporin compound is ISA247.

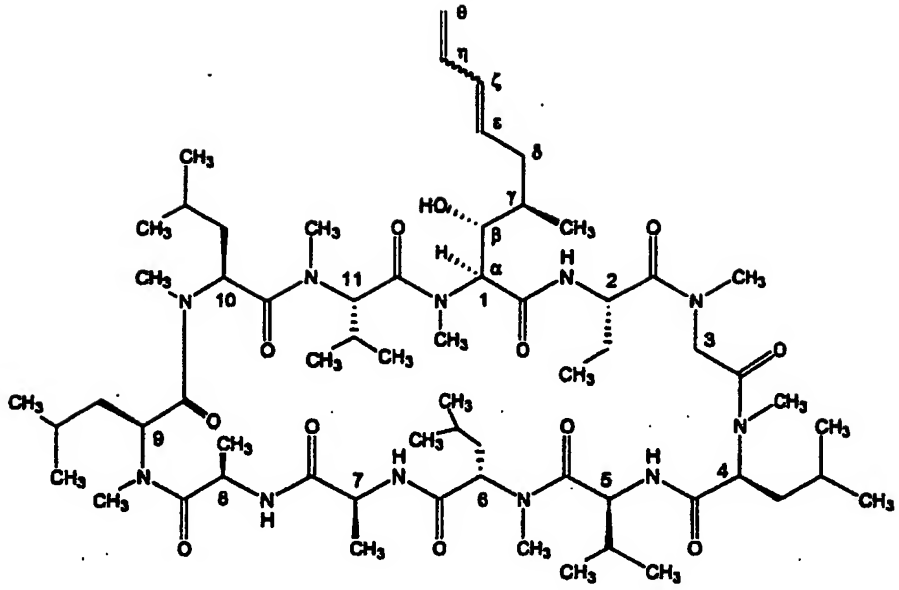
14. The method of claim 12 wherein the cyclosporin compound is cyclosporin A.

15. The method of any one of claims 1-14, wherein the metabolite is selected from the group consisting of IM1-d-1, IM1-d-2, IM1-d-3, IM1-d-4, IM1-c-1, IM1-c-2, IM1-e-1, IM1-e-2, IM1-e-3, IM9, IM4, IM4n, IM6, IM46, IM69, and IM49.

16. The method of any one of claims 1-15 wherein the microorganism is *Saccharopolyspora erthyraea* (ATCC 11635).

17. The method of any one of claims 1-16 further comprising the step of isolating the metabolite from the culture.

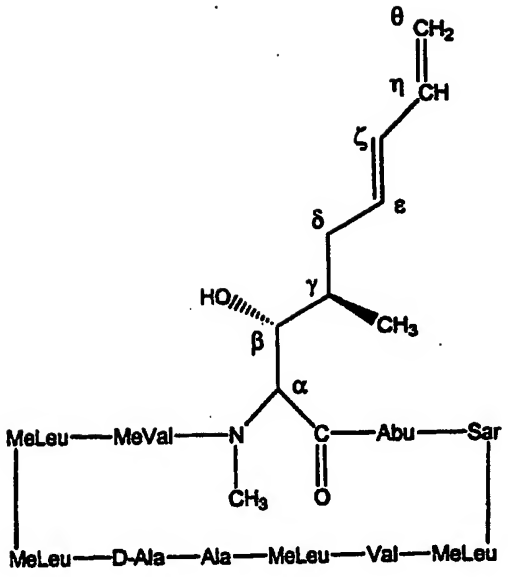
18. A method for identifying a microorganism suitable for use in a biotransformation system comprising: a) comparing the structure of a compound to be metabolized with a known enzyme activity; b) identifying an enzyme that expresses the known enzyme activity; c) identifying a microorganism that expresses the identified enzyme; and d) using the microorganism that expresses the identified enzyme in a biotransformation system to make metabolites of the compound.



ISA 247

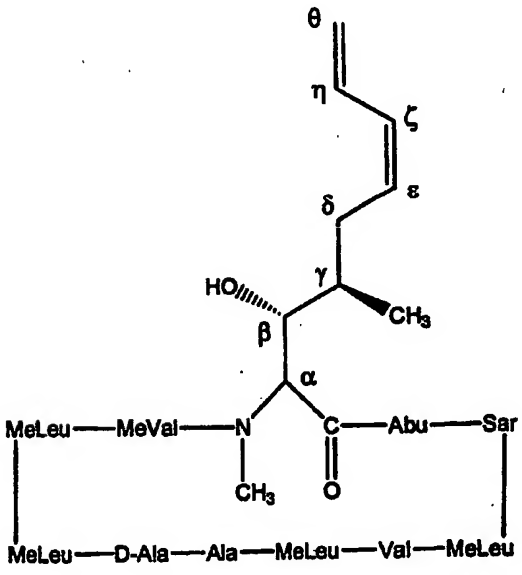
FIGURE 1

FIGURE 2A



E-isomer of ISA 247

FIGURE 2B



Z-isomer of ISA 247

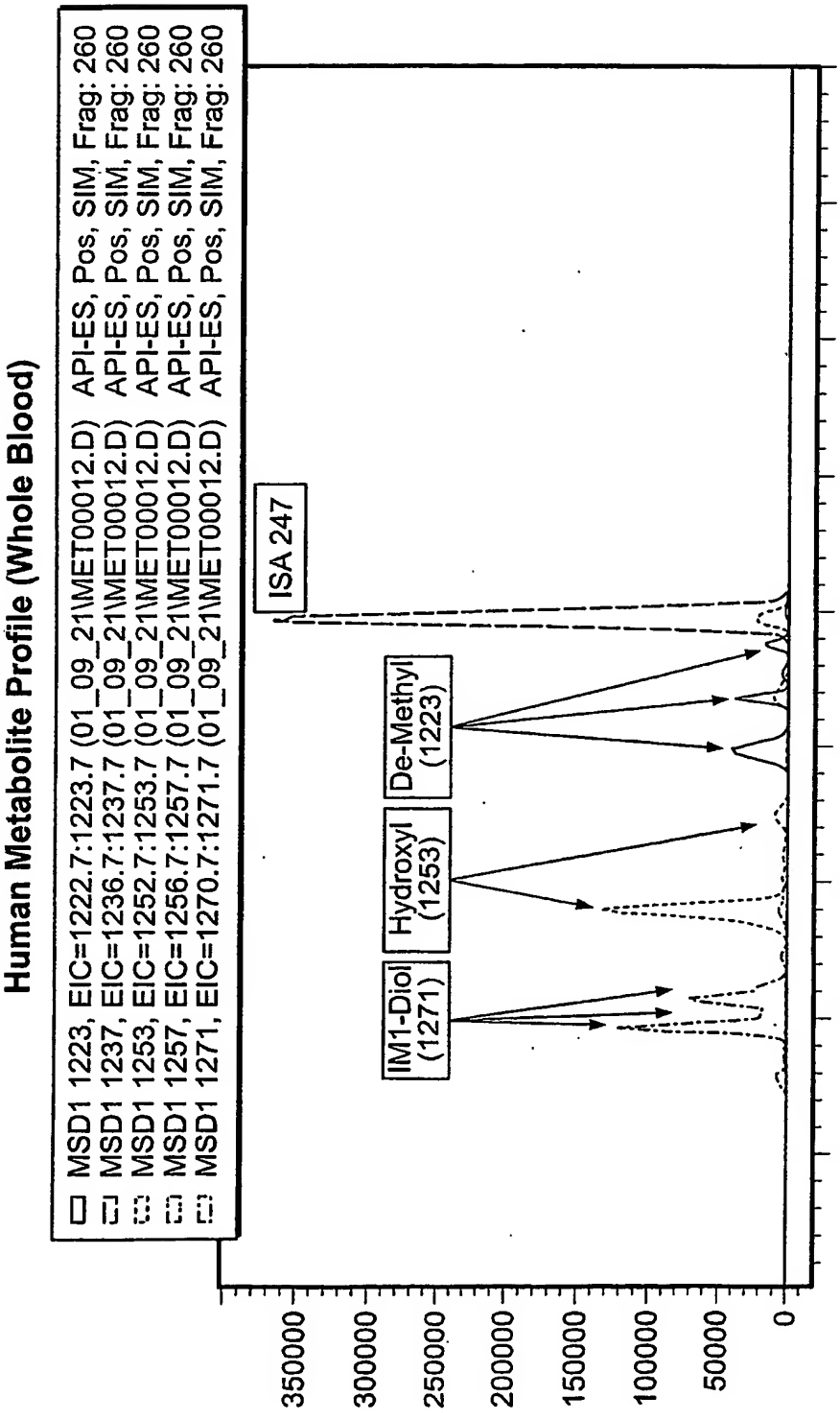

Human Metabolite Profile (Whole Blood)
MSD1 1223, EIC=1222.7:1223.7 (01_09_21\MET00012.D) API-ES, Pos, SIM, Frag: 260
MSD1 1237, EIC=1236.7:1237.7 (01_09_21\MET00012.D) API-ES, Pos, SIM, Frag: 260
MSD1 1253, EIC=1252.7:1253.7 (01_09_21\MET00012.D) API-ES, Pos, SIM, Frag: 260
MSD1 1257, EIC=1256.7:1257.7 (01_09_21\MET00012.D) API-ES, Pos, SIM, Frag: 260
MSD1 1271, EIC=1270.7:1271.7 (01_09_21\MET00012.D) API-ES, Pos, SIM, Frag: 260
ISA 247
IM1-Diol (1271)
Hydroxyl (1253)
De-Methyl (1223)
350000
300000
250000
200000
150000
100000
50000
0
5
10
15
20
25
30
35
40
Minutes

FIGURE 3

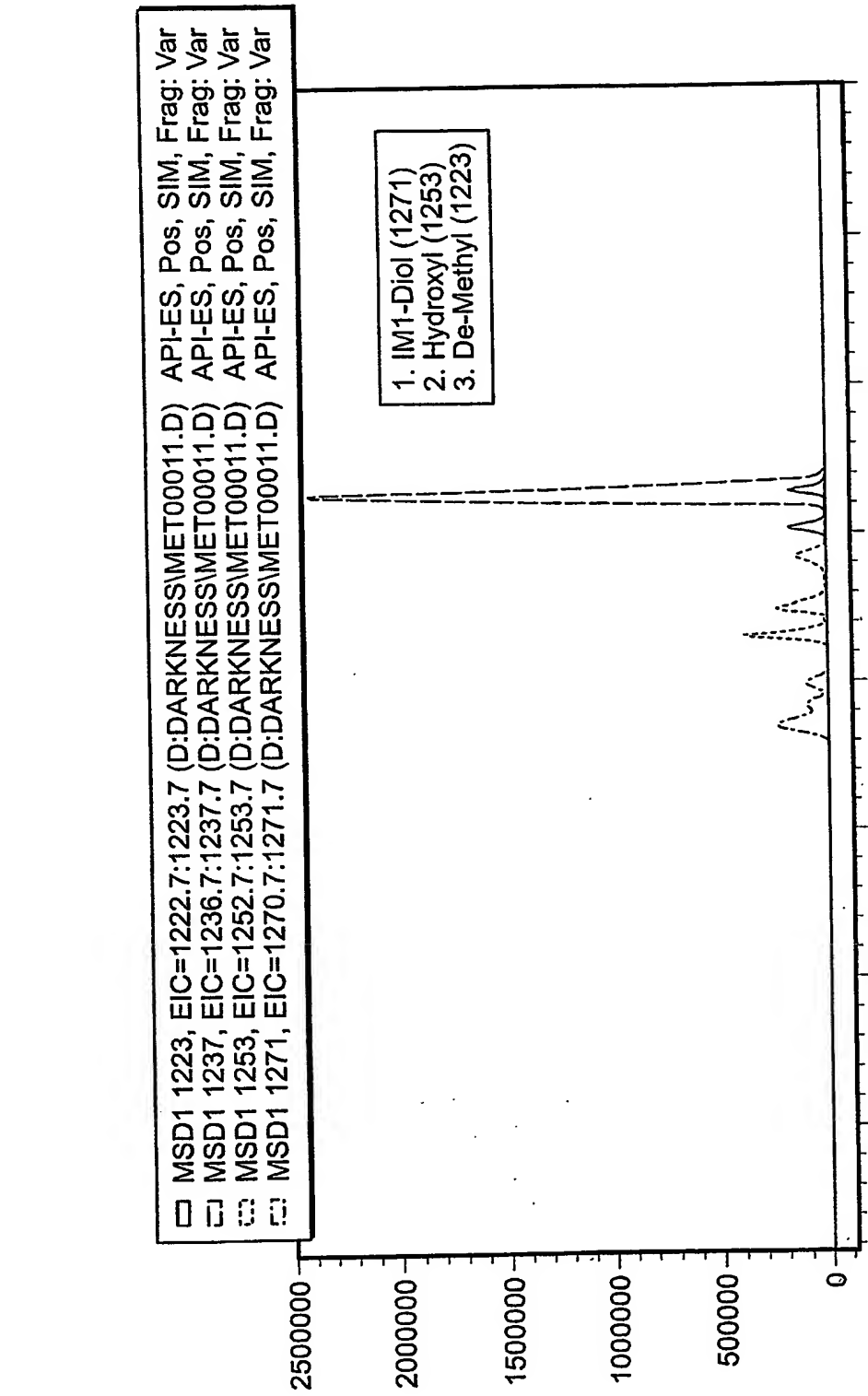

MSD1 1223, EIC=1222.7:1223.7 (D:DARKNESS\MET00011.D) API-ES, Pos, SIM, Frag: Var
MSD1 1237, EIC=1236.7:1237.7 (D:DARKNESS\MET00011.D) API-ES, Pos, SIM, Frag: Var
MSD1 1253, EIC=1252.7:1253.7 (D:DARKNESS\MET00011.D) API-ES, Pos, SIM, Frag: Var
MSD1 1271, EIC=1270.7:1271.7 (D:DARKNESS\MET00011.D) API-ES, Pos, SIM, Frag: Var
1. IM1-Diol (1271)
2. Hydroxyl (1253)
3. De-Methyl (1223)
2500000
2000000
1500000
1000000
500000
0
2
4
6
8
10
12
14
Minutes

FIGURE 4

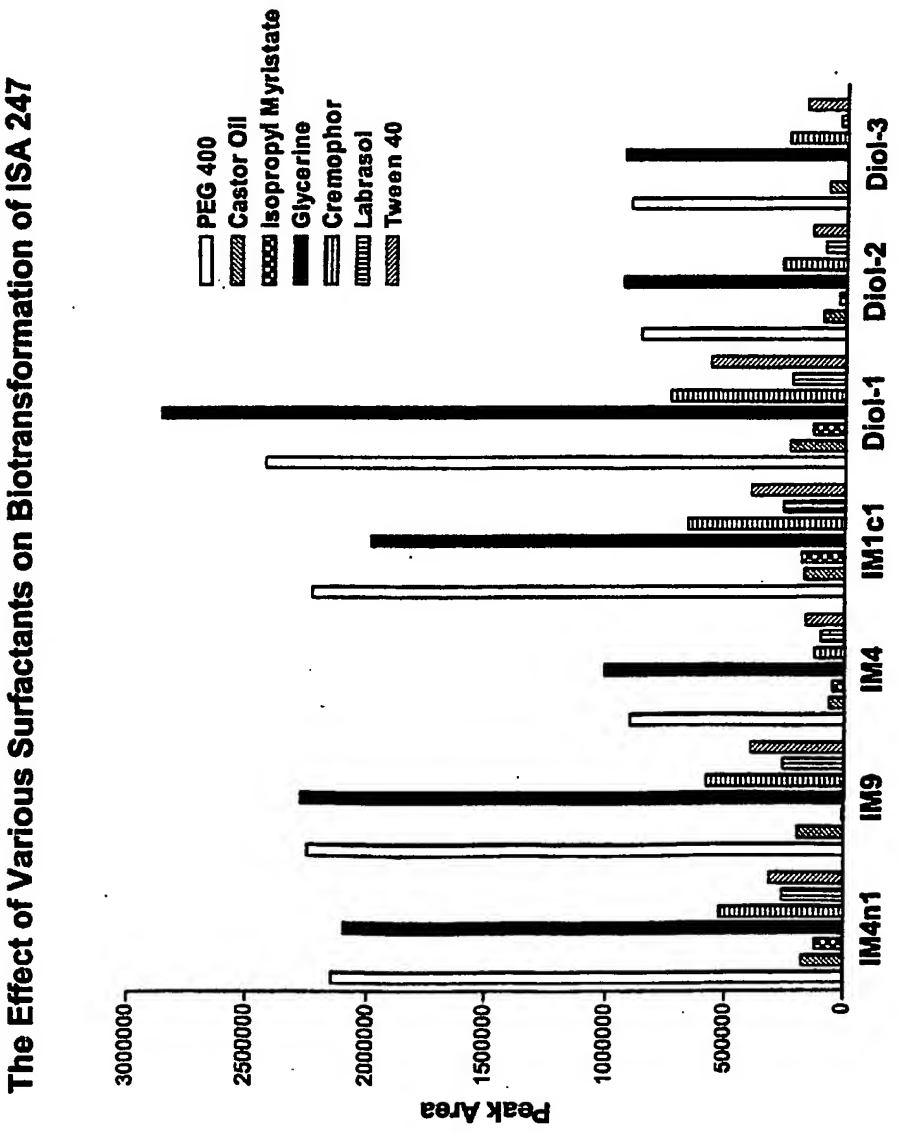

The Effect of Various Surfactants on Biotransformation of ISA 247
PEG 400
Castor Oil
Isopropyl Myristate
Glycerine
Cremophor
Labrasol
Tween 40
Peak Area
3000000
2500000
2000000
1500000
1000000
500000
0
IM4n1
IM9
IM4
IM1c1
Diol-1
Diol-2
Diol-3

FIGURE 5

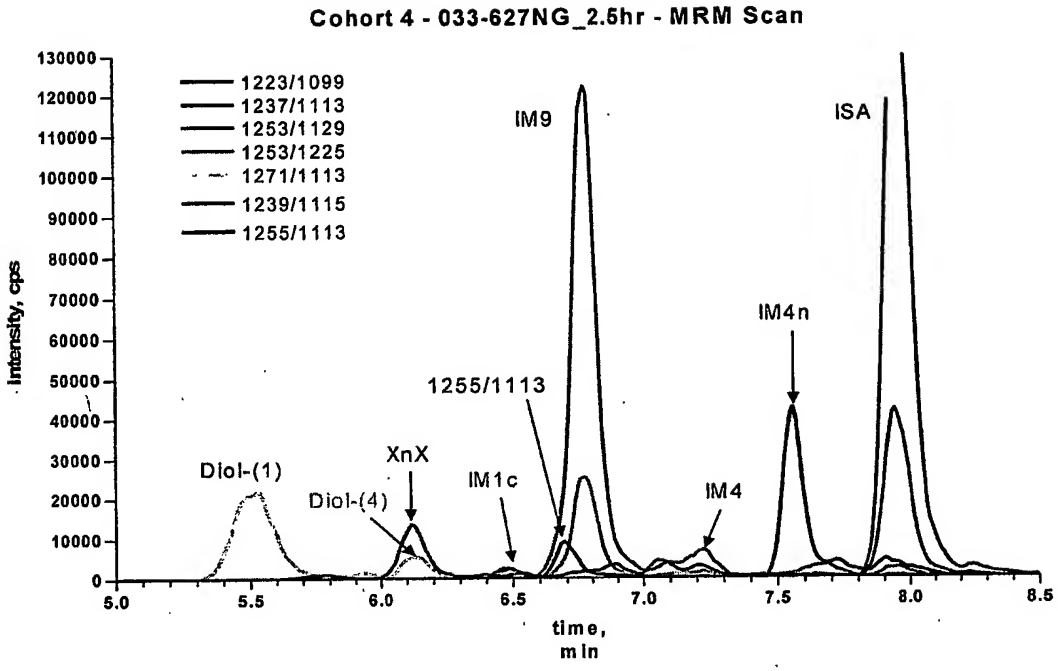

Cohort 4 - 033-627NG_2.5hr - MRM Scan
Intensity, cps
time, min
1223/1099
1237/1113
1253/1129
1253/1225
1271/1113
1239/1115
1255/1113
Diol-(1)
Diol-(4)
XnX
IM1c
1255/1113
IM9
IM4
IM4n
ISA

FIGURE 6

The Effect of Different Types of Fermentation Media on ISA247 Metabolite Production from *Actinoplanes sp.*, ATTC # 53771

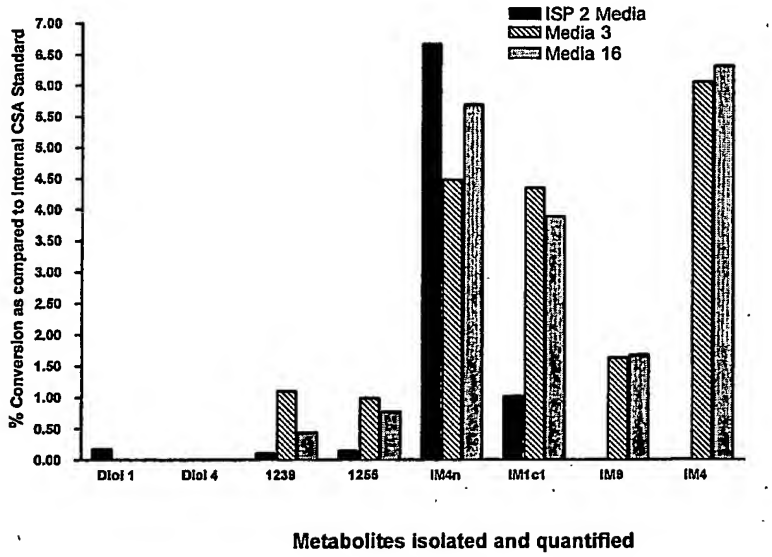


FIGURE 7

The Effect of Different Types of Fermentation Media on ISA247 Metabolite Production from *S. erythraea*, ATTC # 11635

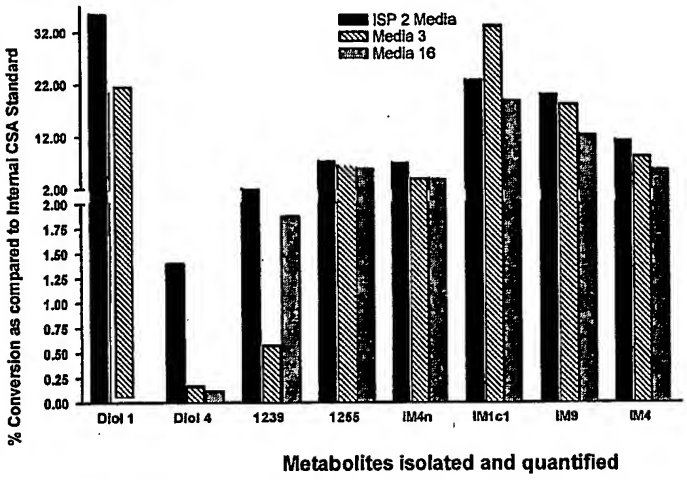
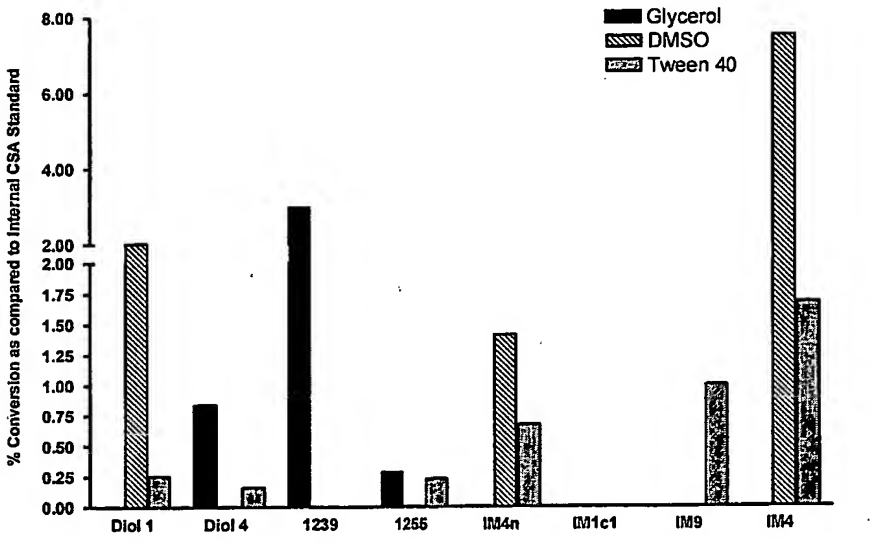


FIGURE 8

The Effect of Different Types of Surfactants to Deliver ISA247 into Fermentation Media for Metabolite Production from *B. bassiana, UAMH # 8717*



Metabolites isolated and quantified from the fermentation of ISA247 with *B. bassiana* (UAMH # 8717)

FIGURE 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001972

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C12P 21/04*** (2006.01), ***C12Q 1/25*** (2006.01), ***C12Q 1/04*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***C12P 21/04*** (2006.01), ***C12Q 1/25*** (2006.01), ***C12Q 1/04*** (2006.01)

CPC: 195/122.6, 195/128.1-199/128.7, 150/15, 150/16.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Canadian Patent Database, Delphion, Derwent, MEDLINE, BIOSIS, BIOTECHNO, CABA, CAplus, EMBASE, ESBIOBASE, FEDRIP, KOSMET, PASCAL, SCISEARCH.

Keywords: biotransformation, surfactant, Tween™, Nonidet™, Chremophor™, Lutrol™, Labrasol™, glycerine, glycerol, cytochrome p450, CYP3A4, metabolite, cyclosporin, ISA247.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YOU et al. "Anaerobic DDT biotransformation: enhancement by application of surfactants and low oxidation reduction potential." Chemosphere	1-5, 10-14 and 17
Y	1996 vol. 32, no. 11, pages 2269-2284 ISSN: 0045-6535 abstract Figures 3 and 4 Table 2 page 2271, last paragraph page 2273, lines 4-8 page 2275, third paragraph - page 2276, end of first paragraph	6-9 and 16

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 March 2006 (28-03-2006)	12 April 2006 (12-04-2006)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001(819)953-2476	Authorized officer John Buchko (819) 953-5926

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001972

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [ ] Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :

2. [ ] Claim Nos. :
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :
because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. Claims 1-17 relate to methods of biotransforming a xenobiotic compound wherein the compound is mixed with a surfactant before being added to a culture media to be biotransformed.

2. Claim 18 relates to a method for identifying a microorganism which is suitable for the biotransformation of a compound.

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001972

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 1483815 A (SHANGHAI HEALTH CREATION CENTER FOR BIOPHARMACEUTICAL R&D CO., LTD.) 24 March 2004 pages 4-5, Table 1 & CN 1483815 A (SHANGHAI HEALTH CREATION CENTER FOR BIOPHARMACEUTICAL R&D CO., LTD.) 24 March 2004 (abstract) World Patents Index [online]. London, U.K.: Derwent Pulblication LTD. [retrieved on 13 March 2006]. Retrieved form: Delphion, Lisle, Il, USA. Accession No. 2004-412235.	1, 10 and 17
X	CA 2390572 A1 (VALIO LTD, FI) 25 May 2001 Abstract	1 and 17
Y	page 6, lines 17-35 Examples 1 and 2 Table 2 Claims 1-3, 7, 8, 10 and 11	6-9 and 16
X	KIEHLMANN et al. "The biotransformation of chrysene to trans-1, 2-dihydroxy-1,2-dihydrochrysene by filamentous fungi." Can. J. Microbiol. 1996	1 and 17
Y	vol. 42, no. 6, pages 604-608 ISSN: 0008-4166 abstract Figures 1 and 2 page 605, left column, paragraph 3	6-9 and 16
X	URLACHER and SCHMID. "Biotransformations using prokaryotic P450 monooxygenases." Current Opinion in Biotechnology	18
Y	1 Dec. 2002 vol. 13, no. 6, pages 557-564 ISSN: 0958-1669 abstract page 557, left column, line 1 - page 557, right column, line 12 Figure 1	6-9 and 16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA2005/001972

Patent Document Cited In Search Report	Publication Date	Patent Family Member(s)	Publication Date
CN1483815 A	24-03-2004	WO2005014845 A1	17-02-2005
CA2390572 A1	25-05-2001	AU770113 B2	12-02-2004
		AU1709301 A	30-05-2001
		BR0015663 A	23-07-2002
		CN1391613 A	15-01-2003
		CZ20021637 A3	13-11-2002
		EE200200253 A	16-06-2003
		EP1240348 A1	18-09-2002
		FI108730B B1	15-03-2002
		HR20020427 A1	31-08-2003
		HU224704 B1	30-01-2006
		JP2003514535T T	22-04-2003
		NO20022361 A	10-07-2002
		NZ518902 A	25-07-2003
		PL355658 A1	04-05-2004
		RU2265664 C2	10-12-2005
		US6960456 B1	01-11-2005
		WO0136653 A1	25-05-2001
		ZA200203539 A	04-08-2003

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